Antithrombotic agents under flow conditions

Thesis

ANTITHROMBOTIC AGENTS

UNDER FLOW CONDITIONS

A thesis submitted in part fulfilment of the requirements for

The Open University degree of Doctor of Philosophy

in the discipline of Life Sciences

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ABSTRACT

Haemostasis is the result of a fine balance of interactions between the endothelium, plasma proteins and blood cells under a wide range of flow rates. To mimic these conditions *in vitro*, a parallel plate flow chamber with human umbilical vein endothelial cells (HUVEC) or extracellular matrix (ECM) has been developed. A method to investigate thrombin generation (TG) in platelet rich defibrinated plasma was validated and used to investigate inhibition by two distinct classes of antithrombotic agents: anti-platelet antibodies and anticoagulants, including unfractionated heparin (UFH), low molecular weight heparin (LMWH) and hirudin.

Increasing flow rates increased TG, which was higher in the presence of ECM than in the presence of HUVEC. All antithrombotic agents investigated were less effective in the presence of ECM.

The monoclonal anti-platelet glycoprotein (GP) IIb/IIIa antibody, RFGP56, partially inhibited TG under static or arterial flow conditions and was less effective under venous flow conditions. The monoclonal anti-platelet GP Ibα antibody, RFGP37, did not inhibit TG under flow or static conditions. A combination of the two antibodies showed no further activity than RFGP56 alone.

UFH, which has equal anti-factor Xa and anti-thrombin activity, was able to inhibit TG under static and flow conditions. On an anti-Xa unit basis, comparatively more LMWH (with a 10:1 ratio of anti-factor Xa to anti-thrombin activity) was required to inhibit TG under static and venous conditions, but under arterial conditions LMWH was as effective as UFH. Hirudin, a thrombin specific inhibitor, was totally effective under static conditions, but was only able to inhibit up to 40 % of TG under flow.

This study shows that some anti-platelet agents can inhibit coagulation and this may contribute to their antithrombotic efficacy under certain flow conditions. Although both the anti-factor Xa and anti-thrombin activities of anticoagulants are effective, anti-factor Xa activity may play a more important inhibitory role under flow conditions.

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PUBLICATIONS

The following publications have resulted from the work included in this thesis.

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Thomas S, (2002) Platelet membrane glycoproteins in haemostasis. *Clinical Laboratory* **48**: 247 – 262.

Thomas S, Rigsby P, and Gray E (2003) The relative importance of anti-Xa and anti-IIa activities in the inhibition of thrombin generation under flow conditions. **Manuscript in preparation**.

Thomas S, Rigsby P, Goodall A H and Gray E (2003) Platelet-mediated thrombin generation in plasma flowing over endothelial cells or their extracellular matrix: involvement of GP IIb/IIIa. **Manuscript in preparation**

The following abstracts, resulting from work included in this thesis, have been published and presented at scientific congresses.

Thomas S, Metcalfe P, Goodall A H and Gray E (1997) Effect of anti-IIb/IIIa antibody and fragment on platelet-mediated thrombin generation. *Thrombosis and Haemostasis* **Suppl**. Poster presentation at the XVIth Congress of the International Society on Thrombosis and Haemostasis.

Thomas S, Metcalfe P, Goodall A H and Gray E (1998) Inhibition of platelet mediated thrombin generation by monoclonal antibodies against GPIIb/IIIa and GPIbα.. *Haemostasis*, **28 Suppl 2** Oral presentation at the 15th International Congress on Thrombosis.

Thomas S, Metcalfe P, Rigsby P, Goodall A H and Gray E (1999) Platelet-dependent thrombin generation in the presence of HUVEC and flow: effects of antibodies to GP IIb/IIIa and GP Ibα. *Thrombosis and Haemostasis* **Suppl** Featured poster presentation and Young Investigator Award at XVII Congress of the International Society on Thrombosis and Haemostasis.

Thomas S, Metcalfe P, Rigsby P, Goodall A H and Gray E (2000) Inhibitory effects of antibodies to GP IIb/IIIa and GP Ibα on platelet-dependent thrombin generation in the presence of HUVEC or ECM and flow. *Haemostasis* **30 (Suppl 1)** Oral presentation at the 16th International Congress on Thrombosis.

Thomas S, Rigsby P and Gray E (2001) Thrombin generation under flow conditions – effect of unfractionated and low molecular weight heparin. *Thrombosis and Haemostasis* **Suppl** Poster presentation at the XVIII Congress of the International Society on Thrombosis and Haemostasis.

Thomas S, Goodall AH and Gray E (2001) Influence of flow on inhibition of platelet dependent thrombin generation by anti-GPIIb/IIIa and GPIbα antibodies. *Blood Coagulation and Fibrinolysis* **12 (7)** Oral presentation at British Society for Haemostasis and Thrombosis Autumn meeting.

Thomas S, Rigsby P and Gray E (2002) The relative importance of anti-Xa and anti-IIa activities in the inhibition of thrombin generation under flow conditions. *Pathophysiology of Haemostasis and Thrombosis* **32 (Suppl 2)** Oral presentation, and co-chair of session at 17th International Congress on Thrombosis.

Thomas S, Rigsby P, Goodall A H and Gray E (2003) Influence of flow on inhibition of platelet dependent thrombin generation by an anti-GP IIb/IIIa antibody. *Journal of Thrombosis and Haemostasis* **Suppl 1** Poster presentation at the XIX Congress of the International Society on Thrombosis and Haemostasis.

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ABBREVIATIONS

α_1AT	α_1-Antitrypsin
α_2M	α_2-Macroglobulin
µg	Micrograms
µl	Microlitre
µM	Micromoles/litre
°C	Degrees centigrade
AcAP	*Ancylostoma caninum* anticoagulant peptide
ACD-A	Acid citrate dextrose formula A anticoagulant solution
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
Anti-IIa	Inhibitory activity against thrombin (usually referring to heparin)
Anti-Xa	Inhibitory activity against coagulation factor Xa (usually referring to heparin)
APC	Activated Protein C
APTT	Activated partial thromboplastin time
AT	Antithrombin
ATP	Adenosine triphosphate
ATU	Anti-thrombin units, thrombin inhibitory units (referring to the activity of hirudin)
AUC	Area under the curve
BSS	Bernard-Soulier syndrome
Ca^{2+}	Calcium ions
CaMKII	Calmodulin-dependent kinase II
cAMP	Cyclic adenosine monophosphate
cGMP	Cyclic guanosine monophosphate
C1-inh	C1 inhibitor
CS	Chondroitin sulphate
CTAD	Citrate, theophylline, adenosine and dipyridamole anticoagulant solution
CV	Coefficient of variance
Da	Daltons
DIC	Disseminated intravascular coagulation
DS	Dermatan sulphate
DVT	Deep vein thrombosis
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbant assay
EPCR	Endothelial cell Protein C receptor
ERK	Extracellular-signal regulated protein kinase
ETP	EDTA, theophylline and prostaglandin E_1 anticoagulant solution
$F(ab')_2$	Bivalent antigen-binding fragment
Fab	Antigen-binding fragment
Fc	Crystallisable fragment of antibody
FII	Prothrombin; coagulation factor II
FIIa	Thrombin; activated coagulation factor II

FITC	Fluorescein isothiocyanate conjugated
FIX	Coagulation factor IX
FIXa	Activated coagulation factor IX
FIXai	Active site inhibited activated coagulation factor IX
FV	Coagulation factor V
FVa	Activated coagulation factor V
FVII	Coagulation factor VII
FVIIa	Activated coagulation factor VII
FVIIai	Active site inhibited activated coagulation factor VII
FVIII	Coagulation factor VIII
FVIIIa	Activated coagulation factor VIII
FX	Coagulation factor X
FXa	Activated coagulation factor X
FXI	Coagulation factor XI
FXIa	Activated coagulation factor XI
FXII	Coagulation factor XII
FXIIa	Activated coagulation factor XII
FXIII	Coagulation factor XIII
FXIIIa	Activated coagulation factor XIII
g	Gravity
GAG	Glycosaminoglycan
gla	γ-carboxyglutamic acid
GP	Glycoprotein
GT	Glanzmann's Thrombasthenia
HBS	HEPES buffered saline
HC II	Heparin cofactor II
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIT	Heparin-induced thrombocytopaenia
HMWK	High molecular weight kininogen
HRP	Horseradish peroxidase
HS	Heparan sulphate
HSA	Human serum albumin
HSPG	Heparan sulphate proteoglycan
HUVEC	Human umbilical vein endothelial cells
IC_{50}	Concentration of inhibitor required to reduce control activity to 50 %
ICAM-1	Intercellular adhesion molecule-1
IL-1	Interleukin-1
IU	International units
IU.seconds/ml	The units in which area under the curve is calculated
l	Litre
LDL	Low density lipoprotein
LMWH	Low molecular weight heparin
LPS	Lipopolysaccharide, endotoxin
M	Moles/litre
MAb	Monoclonal antibody
mg	Milligram
ml	Millilitre
mM	Millimoles/litre
mRNA	Messenger ribonucleic acid

MW	Molecular weight
NAP	Nematode anticoagulant peptide
NAP-6	Recombinant nematode anticoagulant peptide 6
NFκB	Nuclear factor kappa B
NIBSC	National Institute for Biological Standards and Control
nM	Nanomoles/litre
ns	not statistically significant
NO	Nitric oxide
NOS	Nitric oxide synthase
p	Probability
PAb	Polyclonal antibody
PAF	Platelet activating factor
PAI-1	Plasminogen activation inhibitor-1
PAR	Protease activated receptor
PBS	Phosphate buffered saline
PDGF-B	Platelet derived growth factor-B
PF4	Platelet factor 4
PKA	Prekallikrein activator
pM	Picomoles/litre
PS	Phosphatidylserine
r^2	The square of the coefficient of correlation
RGD	Arginine-glycine-proline sequence of amino acids
rNAP-5	Recombinant nematode anticoagulant peptide 5
rNAPc2	Recombinant nematode anticoagulant peptide c2
rpm	Revolutions per minute
rTAP	Recombinant tick anticoagulant peptide
rTF	Recombinant tissue factor
r-TM	Recombinant thrombomodulin
s	Seconds
s^{-1}	Inverse seconds, the unit of shear rate
sd	Standard deviation
Serpin	Serine protease inhibitor
SIPA	Shear-induced platelet aggregation
SSRE	Shear stress response element
TAFI	Thrombin activated fibrinolysis inhibitor
TAP	Tick anticoagulant peptide
TAT	Thrombin-antithrombin complex
TBS	Tris-buffered saline
TBST	Tris-buffered saline + 0.1 % Tween 20
TF	Tissue factor
TFPI	Tissue factor pathway inhibitor
TM	Thrombomodulin
TNF	Tumour necrosis factor
t-PA	Tissue type plasminogen activator
UFH	Unfractionated heparin
u-PA	Urokinase, urinary-type plasminogen activator
VCAM-1	Vascular cell adhesion molecule-1
VnR	Vitronectin receptor
vWF	von Willebrand Factor

CHAPTER 1

INTRODUCTION

1.1 Haemostasis

Haemostasis is the process that prevents blood loss when the wall of a blood vessel is damaged. A rapid reaction is required to seal the breach and allow repair mechanisms to operate and the constant flow of blood over the wound site means that reactants are constantly being washed away from where they are most needed. Efficient capture and activation mechanisms facilitate a localised coagulation response, followed by inhibitory mechanisms that inactivate reactants downstream in order to prevent excessive and obstructive clotting, and finally mechanisms that break down the clot once tissue repair has been completed. In the absence of injury, the fluidity of the blood is maintained by a number of active processes and dynamic interactions between components of plasma, blood cells and the endothelial cells that line the internal surface of blood vessels.

There are two classic pathways that may be followed for blood coagulation to occur. The intrinsic pathway is the process by which blood clots following contact activation of certain coagulation factors by interaction with 'foreign' surfaces. It is called the intrinsic pathway as all the substances required for coagulation are present, or 'intrinsic', in the blood (Saito, 1994). This is in contrast with the extrinsic pathway of coagulation that is stimulated when damage to the vessel wall causes the exposure of the procoagulant lipoprotein tissue factor (TF) that is 'extrinsic' to the vasculature (Broze, 1994). The intrinsic and extrinsic pathways of coagulation do not operate exclusively, and combine to form the common pathway that ultimately leads to the formation of a fibrin clot.

1.1.1 Intrinsic pathway

The contact activation phase of the intrinsic pathway involves four plasma proteins - factor XII (FXII), factor XI (FXI), prekallikrein (PKA) and high molecular weight kininogen (HMWK; see Figure 1.1). The initial interaction is the adsorption of the zymogen factor XII onto a negatively charged foreign surface where it may become activated by limited proteolysis. PKA and HMWK circulate in the plasma as a complex that is also easily adsorbed onto negatively charged surfaces, where PKA is activated to form kallikrein by activated factor XII (FXIIa). The reciprocal reaction also occurs, with kallikrein being the major activator of FXII, and it is therefore clear that amplification of the coagulant response occurs at this stage. Kallikrein also cleaves HMWK to release bradykinin, a vasoactive peptide that can stimulate endothelial cell mediated vasodilation and prostacyclin and tissue plasminogen activator (t-PA) release from the endothelium (Pearson, 1994; Smith *et al*, 1994; Bachmann, 1994). As well as binding to PKA, HMWK binds to FXI (although with a lower affinity than does PKA) and acts as an anchor holding FXI in close proximity to FXIIa, which cleaves the zymogen FXI to the active enzyme FXIa. Following the contact activation phase of the intrinsic pathway, FXIa activates factor IX (FIX) to FIXa that may then combine with activated factor VIII (FVIIIa) on a phospholipid surface in the presence of calcium to form the 'tenase' complex that cleaves and activates factor X (FX) at the start of the common pathway (Saito, 1994).

Patients with a deficiency of one of the four proteins involved in the contact activation pathway tend not to have severe bleeding problems, with the exception of FXI which can lead to variable bleeding tendencies (Bolton-Maggs, 2000). However, the absence or inhibition of these proteins leads to prolonged clotting times

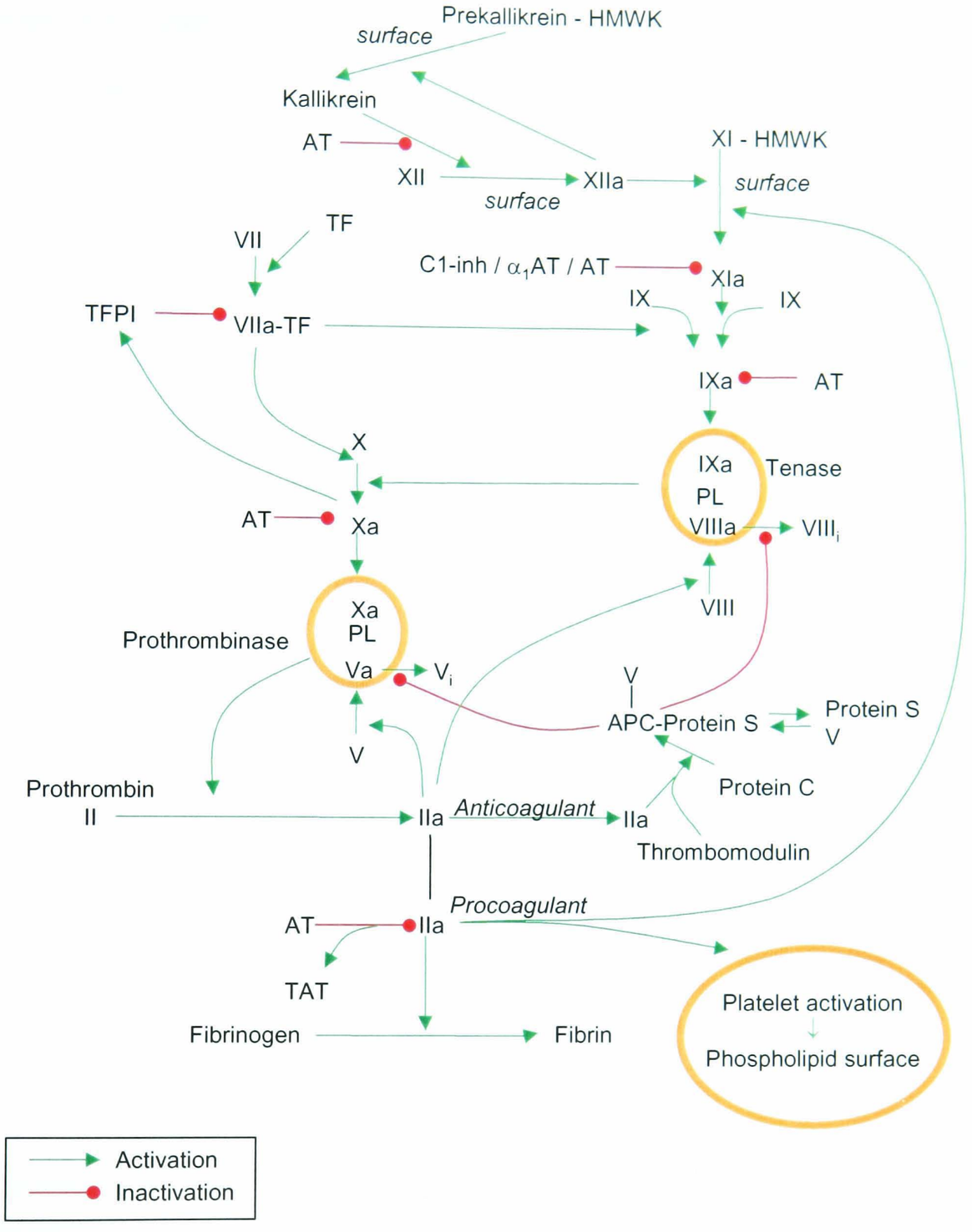


Figure 1.1 *The coagulation cascade.*

Adapted from Sidelmann (2000) and Saito (1994).

Abbreviations are as detailed on page xix with the exception that 'F' is omitted from the clotting factor nomenclature for simplicity.

in the diagnostic activated partial thromboplastin time (APTT) assay, which uses a negatively charged surface (such as a kaolin suspension) to stimulate coagulation. This suggests that there are *in vivo* mechanisms that compensate, such as the feedback activation of FXI by thrombin or a reliance on the extrinsic pathway. Patients that are deficient in FIX or FVIII do have bleeding tendencies (haemophilias B and A respectively), suggesting that although contact activation may be bypassed, the intrinsic coagulation pathway is important for haemostasis. The observation that platelets bind FXII, FXI, FX and HMWK suggests they may play a role in contact activation, possibly providing a catalytic surface for contact activation within the confines of the aggregate (Saito, 1994).

1.1.2 Extrinsic pathway

The principal component of the extrinsic pathway of blood coagulation is tissue factor (TF), a transmembrane lipoprotein constitutively present on the membrane of most extravascular cells. When TF is exposed to blood as a consequence of vessel wall damage, it binds to factor VII (FVII) making it more susceptible to proteolysis by FXa, converting it to the active enzyme FVIIa. The proteolytic activity of the TF-FVIIa complex is much greater than that of FVIIa alone, presumably due to a conformational change that results from binding to TF, which is acting as a cofactor. FVIIa is able to activate FX both directly and indirectly as it is able to cleave FX itself, to give activated factor X (FXa), and also to cleave FIX to FIXa that is then incorporated into the 'tenase' complex, marking the start of the common pathway of coagulation (Broze, 1994).

In addition to its expression by extravascular cells, TF has been detected in leukocytes in circulating blood (Giesen *et al*, 1999; Konigsberg *et al*, 2001) from where it may be transferred to platelets following stimulation (Rauch *et al*, 2000). It remains unclear whether the circulating TF is in an active or cryptic form, or whether it is present in sufficient quantities to initiate coagulation, but the transfer of TF to platelets within a thrombus will facilitate the coagulation process by localising FIXa and FXa, the products of the action of the TF-FVIIa complex, to the phospholipid surface that catalyses their activity (Giesen *et al*, 1999). TF is also expressed by endothelial cells following stimulation with cytokines (see Section 1.3.9).

1.1.3 The common pathway

Once FX is activated, via either the intrinsic or extrinsic pathways, it may itself become incorporated into the prothrombinase complex. The prothrombinase complex consists of FXa and FVa on a phospholipid surface in the presence of calcium, and it cleaves prothrombin (FII) to produce thrombin (FIIa). Thrombin is the major procoagulant protein, and it has numerous procoagulant effects including the activation of the cofactors FV and FVIII and the conversion of fibrinogen to fibrin, which forms the basis of the clot. Factor XIII (FXIII) is a proenzyme that circulates bound to fibrinogen. Once the fibrinogen has been converted to fibrin, thrombin is able to activate FXIII to FXIIIa which then forms covalent bonds between fibrin molecules and cross-links fibrin to other proteins such as collagen and fibronectin, thus stabilising the network of fibrin and strengthening the clot (Ichinose, 2001).

1.1.4 Inhibition of the coagulation process

There are a number of circulating serine protease inhibitors (serpins) of coagulation proteases, such as antithrombin (AT) and heparin cofactor II (HC II), which are dealt with in more detail later in this chapter (see Section 1.3.2). Other relevant serpins include C1-inhibitor (the major plasmatic inhibitor of FXIa, FXIIa and kallikrein), α_1-antitrypsin and α_2-antiplasmin (Wuillemin *et al*, 1996). The other major inhibitor of coagulation proteases is α_2-macroglobulin, which binds to thrombin (Hemker, 1994), FXa (Meijers *et al*, 1987) and kallikrein (Harpel *et al*, 1985).

In addition to these constitutive inhibitors, coagulation stimulates inhibitory mechanisms that 'turn off' the process of coagulation by negative feedback. Although tissue factor pathway inhibitor (TFPI) is a constitutive plasma protein that inhibits coagulation by neutralising the TF-FVIIa complex, it becomes more effective in the presence of FXa once coagulation has proceeded to a sufficient degree (see Section 1.3.4). TFPI may also be found on the endothelial surface, alongside thrombomodulin (TM), which is a receptor for an anionic region of thrombin known as exosite I, where procoagulant substrates of thrombin, such as fibrinogen and FV need to bind to facilitate their presentation to the proteolytic site of thrombin. The binding of thrombin to thrombomodulin therefore blocks its procoagulant activity, but bound thrombin is able to cleave the zymogen Protein C to form the serine protease activated protein C (APC). Together with its cofactor Protein S, APC proteolytically inactivates FVa and FVIIIa (with FV as an additional cofactor), thus effectively switching off the coagulation process (see Section 1.3.3 for more detail).

1.1.5 The current concept of blood coagulation

The relative contributions of the extrinsic and intrinsic pathways of blood coagulation have been outlined by Broze (1995) and Morrissey (2001). The contributions of cell surface phospholipids have been studied by Monroe et al (2002) and the stoichiometry of the initial phase of coagulation modelled by Hockin (2002). It has now been established that coagulation is stimulated via the extrinsic pathway by the exposure of the plasma protein FVII to sub-endothelial membrane-bound TF. This results in the formation of the TF-FVIIa (extrinsic tenase) complex, which then activates FX and FIX, leading to the generation of FXa. The rate of this reaction is governed by the concentrations of FVII and TFPI. In solution, FXa is rapidly inhibited by AT and TFPI but it is protected when bound to cell surface phospholipids, where it also has the ability to cleave prothrombin. The small amount of thrombin generated in this way provides positive feedback activation of FV and platelets, resulting in the assembly of the prothrombinase complex on the platelet membrane and propagation of thrombin generation. This phase lasts 12 seconds in Hockin's model, assuming 5 pM TF as the stimulus for coagulation. As more FXa is generated the inhibitory effect of TFPI becomes more apparent and, for coagulation to continue, the intrinsic tenase complex involving FIXa and FVIIIa (activated by thrombin) must generate FXa. The limiting component in the assembly of prothrombinase is FXa, and the switch in dominance of FX cleavage from extrinsic to intrinsic occurs at around 300 seconds in Hockin's model. The intrinsic tenase complex is fifty-fold more efficient than extrinsic tenase, and peak thrombin generation occurs soon after the intrinsic tenase peak at 600 seconds. The importance of intrinsic tenase is demonstrated by the bleeding tendencies of FVIII and FIX deficient patients. The role of FXI in this scheme is not entirely clear, but it

is possible that its activation by thrombin enables FXIa to activate FIX, maintaining the contribution of the intrinsic pathway (Keularts *et al*, 2001). The dissociation of the A_2 domain of FVIII is the main reason for the decline of intrinsic tenase function, allied to the inhibition of FIXa by AT. In addition to this, the inactivation of FVa by Protein C and of free thrombin by AT and other serpins leads to termination of the coagulant response.

1.1.6 Fibrinolysis

Thrombin also has feedback effects on fibrinolysis, which is the mechanism that breaks down and removes the clot once the damage to the vessel has been repaired. Initially, thrombin inhibits fibrinolysis via the thrombin-activatable fibrinolysis inhibitor (TAFI), an enzyme that interferes with the cofactor activity of fibrin on the activation of plasminogen to plasmin, an enzyme that cleaves fibrin (Bajzar *et al*, 1996). This inhibition may allow the clot to form in the early stages of haemostasis, but following establishment of the clot, the increased amount of thrombin may activate the contact coagulation factors FXII, kallikrein and FXI, enabling them to activate plasminogen. HMWK has also been shown to be required for this reaction. Kallikrein also cleaves plasma pro-urokinase to urokinase (u-PA), which in turn activates plasminogen. The main initiator of fibrinolysis is tissue-type plasminogen activator (t-PA), which is released from endothelial cells activated by exposure to agonists including thrombin (Booth, 1999). Thrombin therefore plays an important role in both the formation and destruction of the clot.

1.2 Platelets

Platelets are disc shaped, non-nucleated blood cells that are usually 2 - 5 μm in diameter. They are formed by the pinching off of sections of the membrane of megakaryocytes, which are large cells found in the bone marrow. The normal count in human blood is in the range 150 – 400 x 10^6 platelets/ml and they have a lifespan of 7 – 10 days. Important structural features of platelets are shown in Figure 1.2 and include i) the glycocalyx, which is comprised of a collection of receptors for adhesion and activation of the platelet; ii) α-granules, the contents of which are released after activation of the platelet; iii) the microtubule and microfilament systems, which lie just inside the platelet membrane and are involved in the shape change process of the activated platelet; iv) the phospholipid membrane that can lose its asymmetry and provide a catalytic surface for coagulation reactions to occur upon; and v) the open canalicular system (OCS), which is a series of interconnected canals formed by invaginations of the membrane, allowing the transport of membrane bound receptors and bound ligands to and from the surface of the platelet.

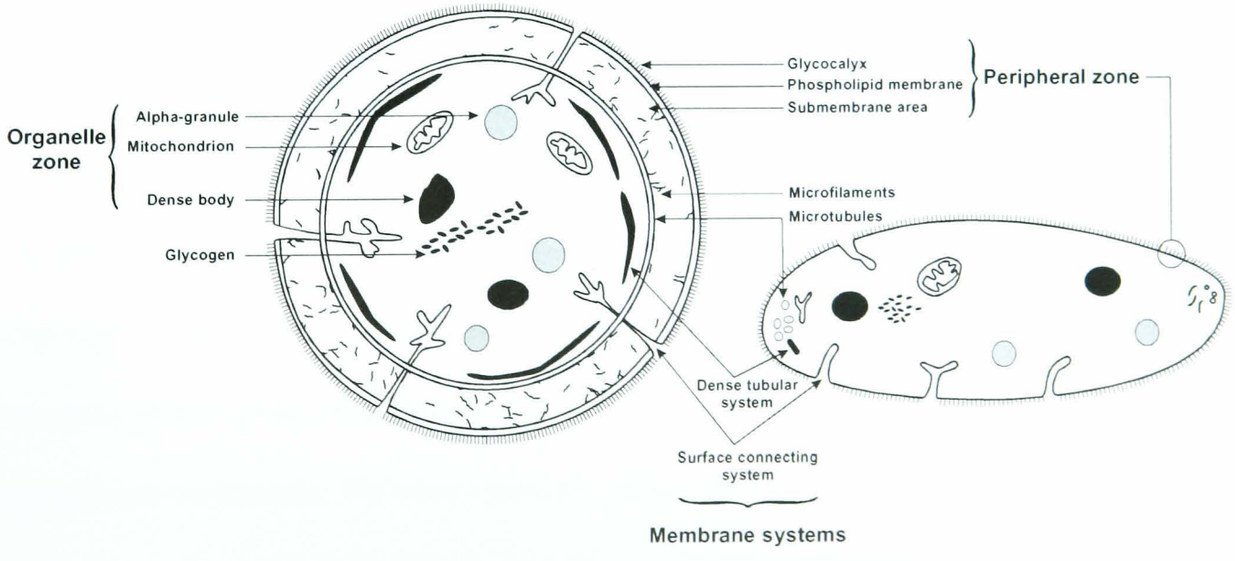


Figure 1.2 *Platelet structure in equatorial and transverse section. Adapted from Kay (1998)*

1.2.1 Platelets and primary haemostasis

Primary haemostasis is the process by which a breach in the vessel wall is sealed, preventing excessive blood loss. Platelets play a critical role, with specialised glycoprotein (GP) receptors enabling them to adhere to proteins exposed in areas of vascular damage. The process of adhesion and/or the interaction of soluble agonists with receptors on the platelet activates the platelets, which are then able to aggregate together to form a platelet plug that seals the breach in the vessel wall. Platelet aggregometry and studies of thrombus growth under flow conditions have determined the platelet activation time to be of the order of 0.1 second (Born & Richardson, 1980). A recent *in vivo* study detected platelet recruitment to the arterial wall 4 seconds after injury, demonstrating the efficiency of the capture and activation mechanisms that lead to the development of a platelet thrombus (Falati *et al*, 2002).

The process by which platelets adhere to areas of vascular damage is a sequence of events that involves a number of receptors and both direct and indirect interaction with the exposed sub-endothelium. The removal of endothelial cells from the lining of the blood vessel exposes a number of proteins that may act as ligands for platelet receptors. The most important is probably collagen, for which platelets have a number of specific receptors, but it is von Willebrand Factor (vWF) bound to the exposed collagen that makes initial contact with the platelet via the GP Ib/IX/V complex (Eldor *et al*, 1985; Sakariassen *et al*, 1986; McCrary *et al*, 1995). Once this contact has been made, the other types of collagen receptors may bind to the exposed collagen, and yet more receptors are able to bind specifically to ligands such as fibronectin, vitronectin and laminin. Some of these receptors are coupled to intracellular signalling systems that lead to the activation of the platelet, and notably

to the conversion to an active conformation of GP IIb IIIa, the receptor that is the main mediator of platelet aggregation.

1.2.1.1 The GP Ib/IX/V complex

The GP Ib/IX/V complex is composed of GP Ibα (CD42b), GP Ibβ (CD42c), GP IX (CD42a) and GP V (CD42d), currently thought to be arranged as shown in Figure 1.3 (Lopez *et al*, 1998), at a density of around 25 000 copies per platelet (Andrews *et al*, 1999). Using platelets from a patient with Bernard-Soulier Syndrome (BSS; a condition where the GP Ib/IX/V complex is deficient) and an antibody that blocked the complex, it was shown that the interaction between the complex and the subendothelium became more significant as shear rates were increased (Sakariassen *et al*, 1986). vWF is an endogenous component of the subendothelium (Sporn *et al*, 1989) that is also adsorbed by collagen and fibrin when they are exposed to flowing blood (Pareti *et al*, 1987; Savage *et al*, 1998; Béguin *et al*, 1999). Three regions of the terminal 282 amino acids of the GP Ibα subunit of the GP Ib/IX/V complex bind to vWF - the leucine rich repeats 2 to 4 (Shen *et al*, 2000), the two disulphide loops formed by 4 cysteine residues at 209, 211, 248 and 264 (Katagiri *et al*, 1990), and three highly sulphated tyrosine residues at positions 276, 278 and 279 (Tait *et al*, 2002). This binding forms a link between the platelet and the extracellular matrix (ECM) under high shear conditions (Weiss *et al*, 1978). This was confirmed with a recombinant fragment of the vWF molecule that competed with vWF for binding to GP Ibα and inhibited subsequent platelet adhesion to ECM (Dardik *et al*, 1993). The GP Ib/IX/V complex is able to bind to surface-bound vWF even when the platelet is moving quickly in flowing blood, facilitated by the macroglycopeptide stalk of GP Ibα that holds the binding regions of the receptor clear of the other cell surface

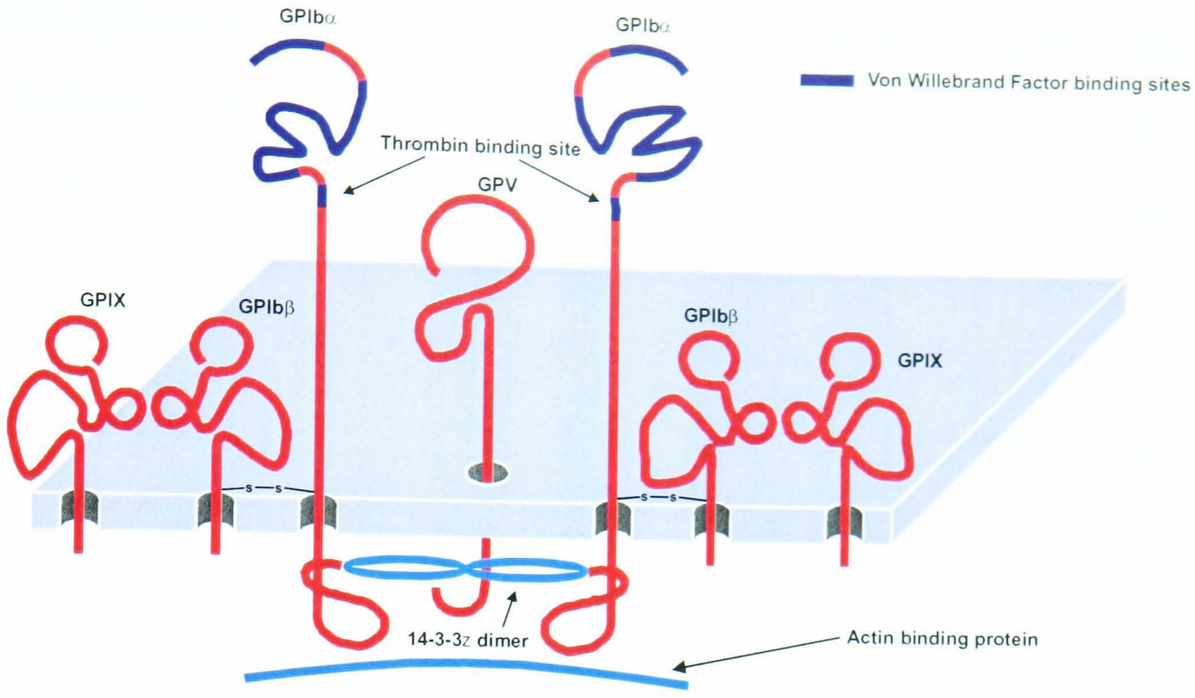


Figure 1.3 *Schematic view of the GP Ib-V-IX complex.*

The thrombin binding site is located between residues 271 – 284, within one of the vWF binding sites. See text for further details.

Adapted from Lopez (1998)

receptors (Li *et al*, 2002) In conditions similar to those found in arterial blood flow (shear rate in the range of 300 – 1000 s^{-1}; see Section 3.1.1 for an explanation of shear rate) the GP Ib/IX/V complex provides the first interaction between platelet and subendothelium and facilitates further, less transient interactions between other ligands and receptors such as GP IIb/IIIa (Frojmovic *et al*, 1997; Kasirer-Friede & Frojmovic, 1998; Sugimoto *et al*, 1999; Wu *et al*, 2000) and GP Ia/IIa (Verkleij *et al*, 1999; Moroi *et al*, 2000). The reason for the mechanistic difference between high and low shear rates has not been established. The conventional theory is that the high shear conditions alter the conformation of the vWF molecule, exposing a site that is able to bind to the GP Ib/IX/V complex. However, two reports suggest that the conformational change induced by high shear may be on the platelet itself, as the exposure of vWF to high shear prior to adhesion experiments under conditions of

low shear did not lead to adhesion (Fredrickson *et al*, 1998; Wu *et al*, 2000). The interaction of GP Ibα and vWF has been demonstrated to lead to the activation of Src kinase in platelets (Wu *et al*, 2002c), indicating a stimulatory pathway for platelet activation.

The GP Ib/IX/V complex also functions as a high affinity receptor for thrombin (Harmon & Jamieson, 1986) and a binding site for thrombin has been identified to be between residues 271 – 284 (De Marco *et al*, 1994), at the top of the macroglycopeptide 'stalk' region of GP Ibα (see Figure 1.3). An additional binding site has also been identified between residues 219 – 240 (McKeown *et al*, 1996) where inhibitory peptides were able to prevent platelet aggregation via GP Ibα or the main thrombin receptor on the platelet membrane, protease activated receptor 1 (PAR-1). Monoclonal antibodies that bind to this region have been shown to inhibit the activation of platelets (LJIb-10; Soslau *et al*, 2001) and the development of platelet procoagulant activity in response to thrombin (SZ2; Dörmann *et al*, 2000). Thrombin cleaves GP V from the surface of the platelet (Lopez & Dong, 1997), possibly following its interaction with GP Ibα, and this may serve to position thrombin in an optimal way to cleave PAR-1 (De Candia *et al*, 2001). It has also been suggested that heparin is able to bind to GP Ibα and inhibit thrombin-mediated platelet activation (Jandrot-Perrus *et al*, 1999), but results from a different group indicate that the inhibitory effect may be due to antithrombin-independent binding of thrombin to heparin (De Cristofaro *et al*, 1998; De Candia *et al*, 1999). GP Ibα has structural similarity to the P-selectin glycoprotein ligand 1 (PSGL-1) on neutrophils and has been demonstrated to be a functional counter-receptor for P-selectin (Romo *et al*, 1999). Unactivated platelets are therefore able to roll on the surface of

activated endothelial cells and to adhere to activated platelets, perhaps initiating thrombosis at sites of inflammation. GP Ibα is also able to bind to Mac-1, a neutrophil integrin that includes a vWF-like I domain in its α subunit, and may therefore recruit inflammatory cells to the growing thrombus.

Signalling via the GP Ib/IX/V complex is summarised in detail by Berndt et al (2001). Under resting conditions, the cytoplasmic tails of the receptor subunits are attached to actin-binding protein (ABP), anchoring the complex to the cytoskeleton, and the adaptor protein 14-3-3 ζ, in association with phosphatidylinositol-3 (PI-3) kinase. Following the interaction of the receptor with an agonist, PI-3 kinase is able to interact with other signalling molecules, such as the Src kinases (Wu *et al*, 2002c), and the receptor complex becomes able to move laterally across the membrane surface. This lateral movement may lead to localisation of GP Ib/IX/V complexes into clusters, where they are re-incorporated into the cytoskeleton, and downstream signalling that may independently activate the platelet (Kasirer-Friede *et al*, 2002). There is controversy in the literature regarding the change in surface expression of the complex following platelet activation. The majority of reports that study platelets in suspension suggest that the complex is temporarily removed to the OCS following platelet activation, in what is possibly a mechanism to down-regulate the platelet's responsiveness to further stimuli (reviewed by Nurden, 1997). However, studies that used adherent platelets appear to give the opposite result, with the complex remaining on the surface of the platelet (reviewed by Escolar & White, 2000).

Functions of the other subunits of GP Ib/IX/V have also been investigated. It has been shown that GP V is involved in slowing down the rolling of the platelet on the

substratum, therefore facilitating other receptor-ligand interactions (Fredrickson *et al*, 1998). GP Ibβ has been shown contribute to the adhesive properties of the complex (Perrault *et al*, 2001) and to be important to the expression (Lopez *et al*, 1992) and stability of the complex (Lopez *et al*, 1994; Moran *et al*, 2000).

1.2.1.2 GP IIb/IIIa

The integrin GP IIb/IIIa ($\alpha_{IIb}\beta_3$; CD41/CD61), shown in Figure 1.4, is a promiscuous receptor that is able to bind a number of ligands, such as fibrinogen, vWF, vitronectin and fibronectin (Beumer *et al*, 1994). This is primarily due to its ability to recognise the Arg-Gly-Asp (RGD) sequence of amino acids, although it is also able to bind to a sequence of 12 amino acids on the γ-chain of fibrinogen (Nurden, 1994). GP IIb/IIIa is usually present on the platelet in a resting form that binds its ligands poorly, but becomes activated following the interaction of a signal-transducing receptor such as GP Ib/IX/V with its ligand. The activation of GP IIb/IIIa by intracellular signalling pathways, reviewed by Shattil *et al* (1998), involves the opening up of the two subunits to reveal the ligand-binding pocket that is concealed within the three dimensional structure of the two subunits. This is often referred to as an 'inside-out' signal. There are reported to be around 80 000 copies of this receptor on the platelet membrane (Wagner *et al*, 1996) and this number may be doubled by the mobilisation of internal stores following activation of the platelet (Michelson & Barnard, 1987; Nurden, 1997). Functional deficiency of GP IIb/IIIa results in Glanzmann's Thrombasthenia (GT), a condition with severe bleeding symptoms that may occur as the result of a wide range of mutations in the genes for either subunit of the receptor (reviewed by French & Seligsohn, 2000).

GP IIb/IIIa plays a role both in adhesion of the platelet to the substratum and in platelet-platelet binding (aggregation). It forms strong bonds with its ligands in the subendothelium but these bonds may only be formed at venous shear rates (below 100 s^{-1}). Where the shear rate is higher, as in arteries (300 – 800 s^{-1}) and capillaries (up to 2800 s^{-1}), this is made possible by the shear-resistant, transient binding of platelet GP Ib/IX/V complexes to vWF. These interactions allow the platelet to roll across the surface at a rate low enough to facilitate binding via GP IIb/IIIa (Savage *et al*, 1992; Frojmovic *et al*, 1997). The main function of GP IIb/IIIa is platelet aggregation, which is achieved by the binding of receptors on multiple platelets to the same multivalent ligand molecule, resulting in the assembly of a platelet plug that produces primary haemostasis, preventing blood loss by sealing breaches in the vasculature.

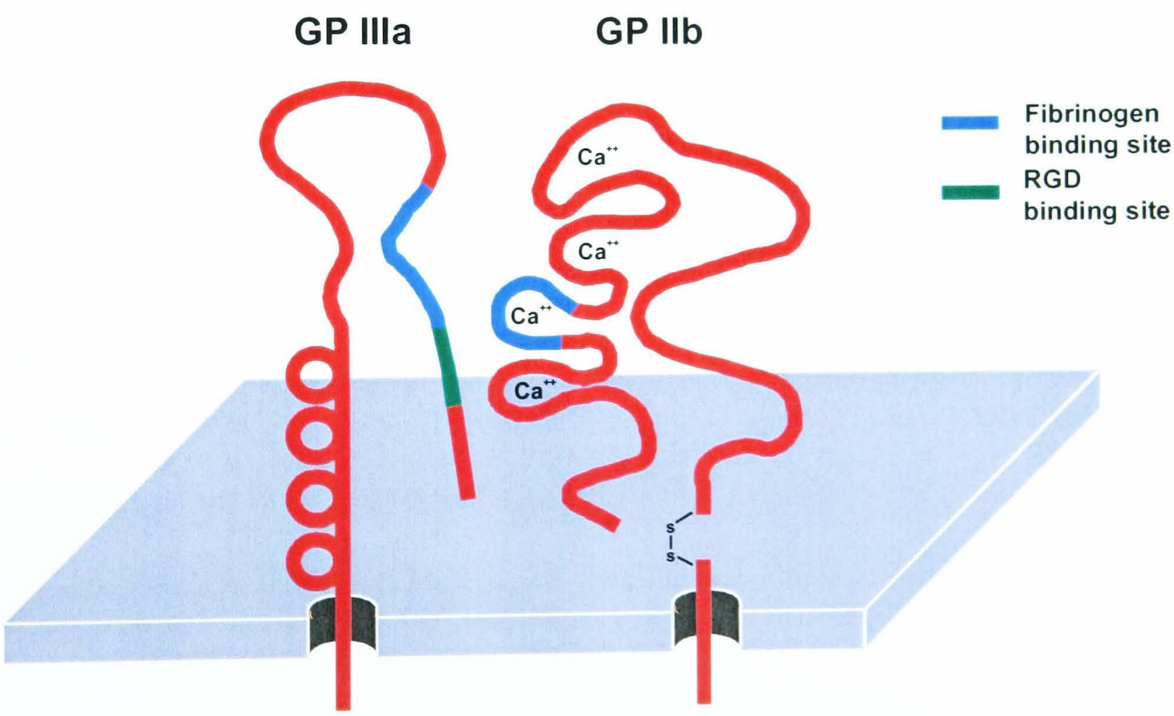


Figure 1.4 *Schematic view of the GP IIb/IIIa receptor. Adapted from Bachelot et al (1995).*

1.2.1.3 Collagen receptors

The receptor currently thought to be most important for direct adhesion of platelets to collagen is GP Ia/IIa ($\alpha_2\beta_1$, CD49b/CD29, VLA-2, see Figure 1.5), another member of the integrin family of receptors. Antibody studies have shown that GP Ia/IIa is able to interact with collagen under flow conditions (Verkleij *et al*, 1999; Siljander & Lassila, 1999) and that inhibition of GP Ia/IIa leads to an increased velocity of platelet rolling across the collagen surface (Moroi *et al*, 2000). This suggests that GP Ia/IIa is involved in the tight binding of tethered platelets to the collagen surface and that activation of the platelet may be occurring as it continues its way across the surface. As the platelet becomes more activated, other receptors will become able to interact with the surface leading to arrest of the platelet.

It has been shown that there are two distinct activation states of GP Ia/IIa, and that the state of the integrin can be influenced by the presence of the soluble platelet agonists ADP and thrombin (Jung & Moroi, 2000). The Ia subunit of GP Ia/IIa is where the specificity for collagen is defined, and it has an I domain insert that has homology with the A domain of vWF, suggesting its suitability for interaction with collagen. The IIa subunit is also important, and studies have shown that antibodies to different epitopes can cause either activation of the integrin or inhibition of its collagen-binding potential, suggesting that the IIa subunit has a role in maintaining the active conformation of the integrin (Moroi & Jung, 1997).

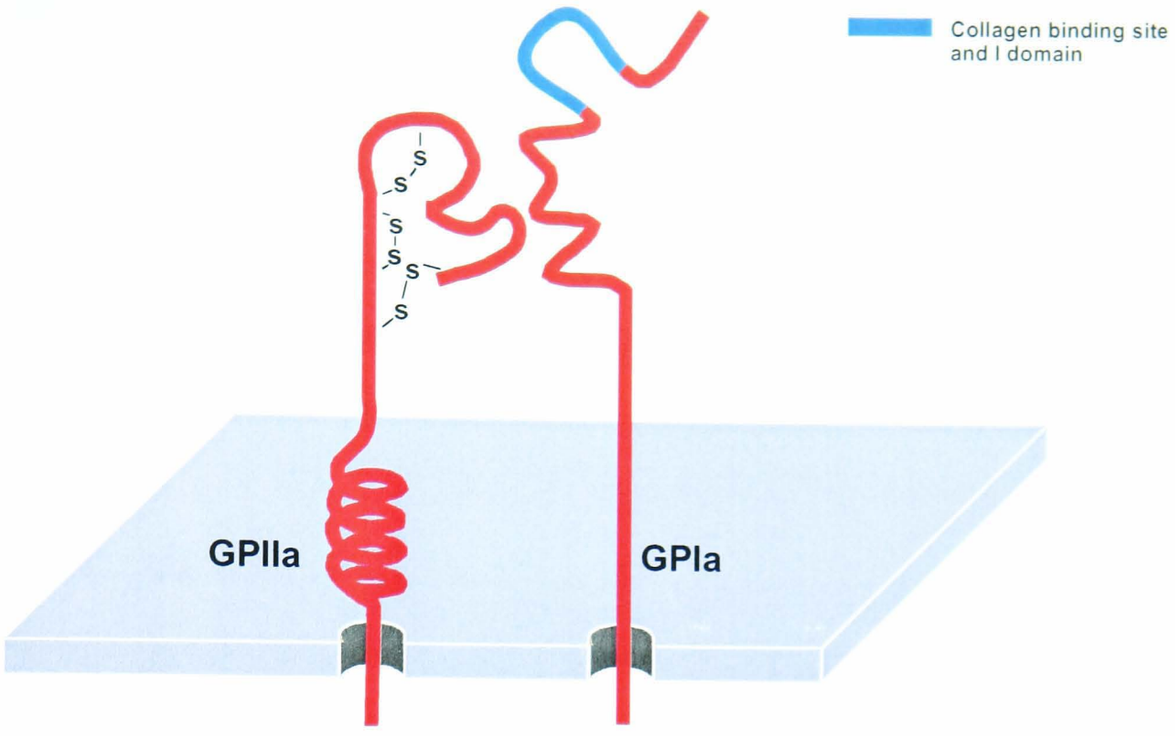


Figure 1.5 *Schematic view of the GP Ia/IIa receptor. Adapted from Santoro and Zutter (1995)*

Glycoprotein VI (P62) has been demonstrated to be a receptor for collagen (Moroi *et al*, 1989; Ishibashi *et al*, 1995) that reacts with an amino acid sequence (glycine-proline-hydroxyproline) that is highly specific to collagens (Knight *et al*, 1999). Deficiency of this receptor does not lead to profound bleeding complications (Moroi & Jung, 1997), probably due to the presence of functional GP Ia/IIa on the platelets (Ichinohe *et al*, 1997). Although this receptor plays only a minor role in adhesion, the activation of tyrosine kinases, phospholipases and focal adhesion kinases following platelet exposure to collagen has been shown to be dependent on GP Ia/IIa, but possibly with the co-operation of GP VI (Keely & Parise, 1996; Ichinohe *et al*, 1997; Kehrel *et al*, 1998). This cooperative action may be related to its significance to the procoagulant response of activated platelets (Heemskerk *et al*, 1999).

Glycoprotein IV (CD36) has been shown to be involved in the binding of platelets to subendothelial surfaces at physiological shear rates (Diaz *et al*, 1996) and to be mobilised to the membrane from internal stores following activation of the platelet (Berger *et al*, 1993; Diaz *et al*, 2000). It has been identified as a receptor for collagen and thrombospondin (Nurden, 1994) but patients who are genetically deficient in this receptor do not present with bleeding problems (Tandon *et al*, 1991).

1.2.1.4 Other adhesive receptors

Platelets possess a number of other receptors for proteins that are found in the subendothelium. Glycoprotein Ic/IIa ($\alpha_5\beta_1$, VLA-5) is an integrin receptor for fibronectin, an adhesive protein that is secreted from platelet α-granules and binds to collagens and other subendothelial proteins in damaged vessel walls. GP Ic/IIa has been shown to support platelet adhesion at high shear rates (Polanowska-Grabowska *et al*, 1999).

Vitronectin is another subendothelial protein, and its integrin receptor (VnR, $\alpha_V\beta_3$) is present in low numbers (around 50 copies) on the platelet membrane (Lam *et al*, 1989). Like GP IIb/IIIa, the vitronectin receptor is a promiscuous receptor able to bind to other RGD-containing ligands such as fibrinogen, fibronectin, and vWF in addition to vitronectin (Cheresh *et al*, 1989). Glycoprotein Ic'/IIa ($\alpha_6\beta_1$, VLA-6) has been shown to be a receptor for laminin, which is a three chain adhesive protein that is found in the subendothelium (Sonnenberg *et al*, 1988). GP Ic'/IIa is able to bind to laminin at shear rates below 1000 s^{-1} (Polanowska-Grabowska *et al*, 1999) but there are no reports to suggest that platelets become activated following binding to laminin via this receptor.

The combination of all these interactions has the effect of firmly anchoring the platelet to the vessel wall, facilitating its next contributions to haemostasis – aggregation and procoagulant activity.

1.2.1.5 Platelet aggregation

The adherence of platelets to the vessel wall, or their interaction with soluble agents such as thrombin and ADP that may be present at the site of tissue damage, leads to the generation of intracellular signals. These are sometimes referred to as 'outside-in' signals, and their common effect is the engagement of the cytoskeleton, and the shape change and spreading of the platelet across the substrate.

The 'inside-out' signal that results from the unmasking of the ligand-binding pocket on the GP IIb subunit of GP IIb/IIIa leads to an increased affinity of the receptor for its ligands and marks a change in the character of the platelet from adhesive to aggregatory. Its multivalent ligands such as fibrinogen and vWF can bind to copies of GP IIb/IIIa on adjacent platelets, creating a cross-linked matrix of platelets and proteins (Shattil *et al*, 1998). This leads to amplification of the response and the recruitment of platelets to the site of damage, forming a platelet plug that is the first line of defence in the prevention of blood loss from damaged blood vessels.

1.2.2 Platelets and secondary haemostasis

The second line of defence in the prevention of blood loss is the formation of a fibrin clot. This clot is built on the scaffold of aggregated platelets that form the platelet plug and there are a number of ways in which platelets facilitate the formation of the clot. Activated platelets undergo a rearrangement of their membrane structure, and

negatively charged phospholipids such as phospatidylserine (PS) become exposed at the outer leaflet (Zwaal *et al*, 1989). This process is often referred to as a 'flip-flop' of the membrane or 'membrane scrambling' and is the result of the action of the enzyme scramblase (Zwaal & Schroit, 1997). The exposed phospholipids provide a catalytic template for the interaction of FIXa and FVIIIa to form the tenase complex, and for the interaction of FXa and FVa to form the prothrombinase complex.

Platelets facilitate these reactions further by carrying some of the relevant enzymes and cofactors on or inside their membrane or on parts of their membrane that become 'pinched off' after activation to form microparticles. Factor VIII, which is a cofactor in the tenase complex, circulates bound to vWF and the local concentration of FVIII will therefore increase at sites of vessel wall damage when vWF binds to collagen exposed in the subendothelium. Factor VIII can then bind to receptors on the platelet membrane, the expression of which is increased following activation of the platelet (Gilbert *et al*, 1991), suggesting that there is an active recruitment of this cofactor to the membrane. Similarly, receptors for FV have been detected on microparticles (Sims *et al*, 1988), and platelets have been shown to synthesise and secrete this coagulation cofactor (Monkovic & Tracy, 1990; Camire *et al*, 1998) showing that the formation of the prothrombinase complex is also facilitated by platelets. Prothrombin, the substrate of this complex, is the zymogen of thrombin, the most potent enzyme of the coagulation cascade. Prothrombin is also carried on the membrane of resting platelets; the relatively small size of the prothrombin molecule allows it to enter the ligand-binding pocket of unactivated GP IIb/IIIa, which is inaccessible to the larger fibrinogen molecule, and bind via its RGD sequence (Byzova & Plow, 1997). It has also been reported that FXI may bind to GP Ib/IX/V

in a way that facilitates its activation by thrombin, but this occurs after platelet activation suggesting that it may be a feedback effect in the propagation phase of coagulation (Baglia *et al*, 2002).

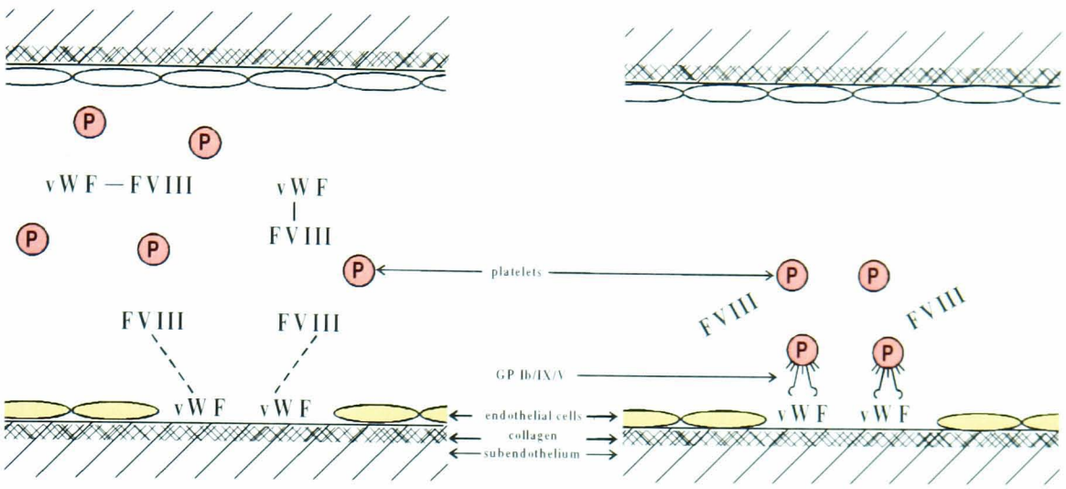
We can therefore envisage a sequence of platelet-mediated haemostatic events that follow damage to the endothelium (illustrated in Figure 1.6). Platelet activation by interaction with subendothelium-bound vWF leads to platelet shape change, membrane 'flip-flop' and the activation of GP IIb/IIIa. As this happens, the cofactor FVIIIa (released from the vWF) binds to the membrane with FIXa to form tenase that cleaves FX to create FXa that then combines with FV released from the platelet, activated by thrombin, and is bound to the platelet membrane to create prothrombinase. Prothrombin is displaced from the now activated GP IIb/IIIa, which can now bind the larger fibrinogen molecule for which it has a higher affinity, and the prothrombin is cleaved to create thrombin that in turn cleaves the fibrinogen to create the fibrin that forms the clot.

1. Endothelial damage

- vWF binds to collagen in the exposed subendothelium.
- factor VIII (FVIII) is released from vWF as it binds.

2. Platelet tethering

- the GP Ib/IX/V complex is able to bind to vWF adsorbed onto the subendothelium under conditions of shear stress.

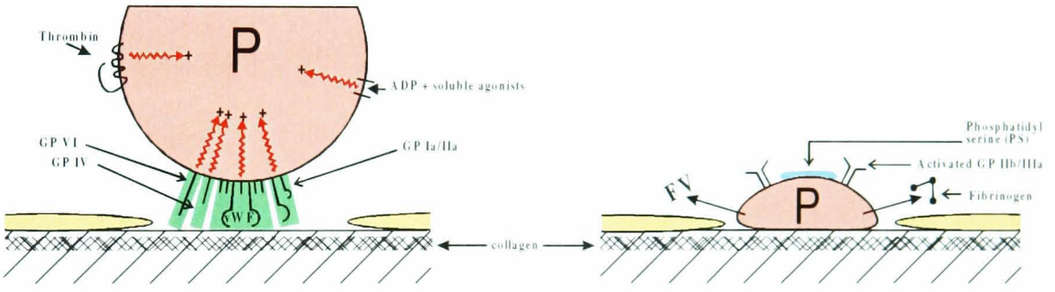


3. Platelet adhesion

- tethering via GP Ib/IX/V allows other receptors to form stronger bonds with their ligands, eg collagen.
- the interaction of signal-transducing receptors with their ligands leads to 'outside-in' signals and platelet activation.

4. Platelet activation

- activated platelets adhere to the subendothelium and spread out.
- fibrinogen and Factor V (FV) are released.
- GP IIb/IIIa becomes activated.
- membrane expresses negatively charged phospholipids (PS).



5. Aggregation

- fibrinogen acts as a bridge leading to the formation of a 'scaffold' of activated platelets.
- the binding of fibrinogen to IIb/IIIa displaces prothrombin (FII).

6. Coagulation

- the negatively-charged phospholipids on the outer leaflet of activated platelets act as catalytic templates for the formation of the 'tenase' and 'prothrombinase' complexes.
- the thrombin (IIa) that is formed is then able to cleave fibrinogen to form fibrin, the basis of a clot, and also to activate factors V and VIII to accelerate coagulation.

Figure 1.6 *A schematic representation of platelet-mediated haemostasis*

1.3 The endothelium and inhibitors of coagulation

The maintenance of a non-thrombotic state in normal blood vessels is largely dependent on the integrity of the endothelium. The endothelium usually presents a non-thrombogenic surface and expresses a variety of anticoagulant factors, although procoagulant activity may be induced.

1.3.1 Inhibition of intrinsic activation

Factor XII and HMWK have been found to compete for a binding site on the surface of endothelial cells, suggesting that the cell membrane may provide a template for the interaction of these molecules and thereby stimulate the intrinsic pathway of coagulation, especially as bound FXII was shown to be gradually cleaved to its active form FXIIa that was able to cleave prekallikrein (Reddigari *et al*, 1993). The assembly of a FIX activating complex on the endothelial cell membrane following the binding of HMWK and FXI in the presence of FXIIa has also been demonstrated (Berrettini *et al*, 1992). Contrary to this, it has been demonstrated that endothelial cells are able to inhibit the formation of FXa and kallikrein in plasma, indicating inhibition of the intrinsic pathway of coagulation in a process independent of Protein C and suggested to be via inactivation of FVIII (Kleniewski & Donaldson, 1993; Bombeli *et al*, 1996; Bombeli *et al*, 2001).

Endothelial cells may also enhance the inhibition of FXIa by the plasmatic inhibitors antithrombin and C1-inhibitor and of kallikrein by antithrombin. *In vitro* the action of both of these inhibitors is enhanced by interaction with heparin (Olson *et al*, 1993; Wuillemin *et al*, 1995) and it has been suggested that glycosaminoglycans (GAG) on

the luminal surface of endothelial cells may provide this potentiation *in vivo*, adding to the inhibitory properties of the endothelium (Wuillemin *et al*, 1996).

1.3.2 Heparan sulphate, heparin and antithrombin

The major antithrombotic component of endothelial cells is the heparan sulphate proteoglycan (HSPG) molecules that are abundant on the cell membrane (Busch & Owen, 1982; Pearson, 1994). These negatively-charged GAG have the ability to potentiate the inhibitory action of a number of inhibitors, such as antithrombin and C1-inhibitor.

1.3.2.1 Antithrombin

Antithrombin (AT) is one member of a group of serine protease inhibitors (serpins) that function by acting as pseudosubstrates for their target enzymes, which become covalently bound and are removed from the circulation. It is able to inhibit a number of coagulation proteases such as FXIa and FIXa but most important is its inhibition of thrombin and FXa, proteases in the common pathway of coagulation (Bauer & Rosenberg, 1991; Harper & Carrell, 1994).

AT is a single chain glycoprotein of 432 amino acids with three disulphide bonds, and a molecular weight of 58 200 (Lane *et al*, 1994). It exists in two isoforms, AT-α with four carbohydrate side-chains and AT-β with three side-chains and an increased affinity for heparin, which leads to greater inhibitory activity (Swedenborg, 1998). AT circulates in the plasma at a concentration of 2.5 μM or 125 μg/ml (Murano *et al*, 1980) and 90 % is present as the AT-α isoform (Peterson & Blackburn, 1985). This is in contrast to the vessel wall where the proportions of AT-α and AT-β are equal.

suggesting that the high-affinity AT-β isoform preferentially binds to heparin-like molecules on the endothelium before being transferred through the endothelium and into the vessel wall (Bock *et al*, 1997).

1.3.2.2 Heparin

The action of antithrombin is greatly enhanced by binding to the polysaccharide heparin, a heterogeneous mixture of polysaccharide chains of varying lengths that is naturally found in the granules of mammalian mast cells in tissues such as lung, skin, ileum, lymph nodes and thymus. No evidence has been found to demonstrate that endogenous heparin plays a role in maintaining blood flow, but it has proved to be an effective therapeutic agent for this purpose and is most commonly prepared from porcine intestinal mucosa (Sarret, 1999). The interaction of heparin with antithrombin leads to a conformational change that exposes the reactive centre of antithrombin, and neutralisation of its positive charge that allows it to interact more easily with other positively charged molecules. These changes lead to a 10 000-fold increase in antithrombin's inhibitory rate against thrombin (Harper & Carrell, 1994). The length and precise composition of each heparin chain influences its biological activity, and enables it to act as a catalyst for the inhibition of FXa and FIIa (thrombin) by antithrombin. The interaction of heparin with antithrombin is highly specific and the sequence that defines this specificity has been identified as a pentasaccharide sequence (Choay *et al*, 1983). Within this sequence, a 3-*O*-sulphate group on the glucosamidine residue in position 3 of the pentasaccharide is critical for the binding of antithrombin (Lindahl *et al*, 1980; see Figure 1.7). Not all of the polysaccharide chains will contain the pentasaccharide sequence, but some long polysaccharide chains may contain several repeats, meaning that the activity of the

heparin mixture is influenced by its molecular weight. The mean molecular weight of an unfractionated heparin (UFH) preparation is around 15 000, representing chains of up to 50 saccharide units.

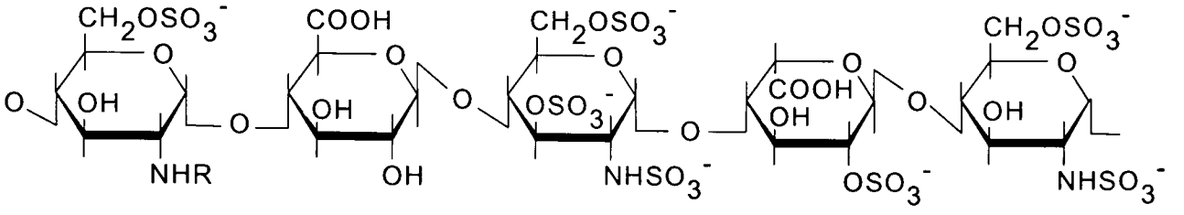


Figure 1.7 *The structure of the heparin pentasaccharide with high affinity for antithrombin. R = Ac or SO_3^- Reproduced from Mulloy & Forster (2000).*

Once antithrombin has bound to heparin, and its binding affinity for FXa therefore increased, FXa is prevented from being incorporated into the prothrombinase complex, inhibiting the action of the complex in the conversion of prothrombin to thrombin. However, for heparin to enhance antithrombin's direct inhibition of thrombin it is necessary for a ternary complex to be formed, with thrombin binding to the heparin chain alongside antithrombin (see Figure 1.8). Thrombin binds to heparin via an anion binding domain known as exosite II (Sheehan & Sadler, 1994; Gan *et al*, 1994), and a chain length of thirteen saccharides is required for this interaction, meaning that a minimum chain length of 18 saccharides is required for the inhibition of thrombin compared to the five required for inhibition of FXa (Lane *et al*, 1984; Danielsson *et al*, 1986). An 18 saccharide chain will have a molecular weight of around 5 400, and this is often referred to as the critical chain length for

potentiation of antithrombin activity against thrombin (Bray *et al*, 1989). The probability of the critical pentasaccharide sequence occurring next to a 13 saccharide sequence increases as the length of the chain increases and it follows that preparations of heparin with higher molecular weight will have a greater anti-IIa activity than low molecular weight preparations. Antithrombin's inhibition of thrombin prevents the formation of fibrin clots and also serves to inhibit the positive feedback thrombin-mediated activation of FV, FVIII, FXIII and platelets.

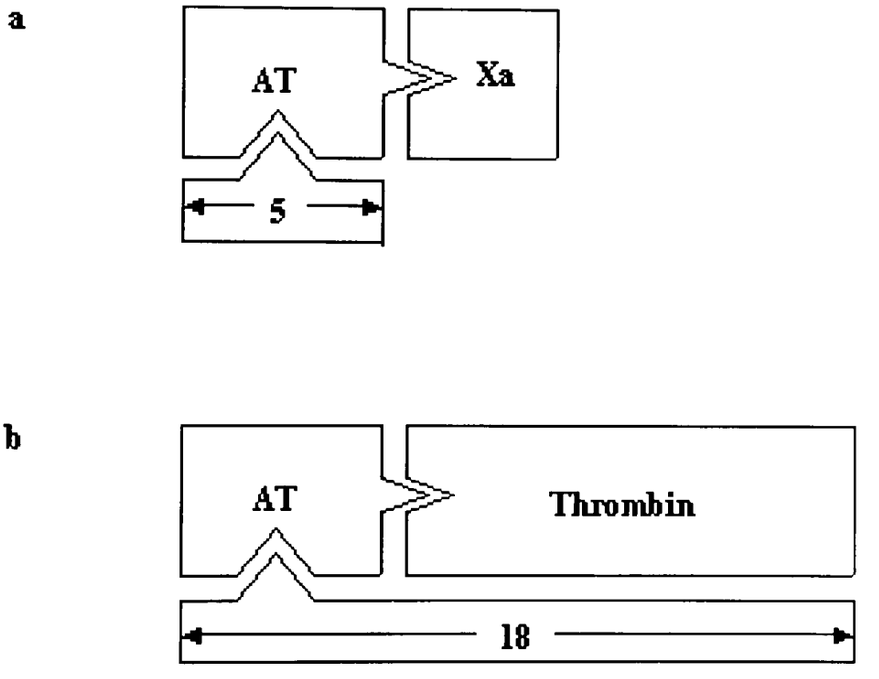


Figure 1.8 *The template model for the potentiation of a) factor Xa and b) thrombin inhibition by heparin.*

The pentasaccharide sequence (5) is required for the interaction of heparin with antithrombin (AT), increasing its affinity for Xa. An additional 13 saccharides are required for the binding of thrombin.

1.3.2.3 Heparin and cells

Heparin binds to two sites on endothelial cells in a slow and saturable manner, although these sites are not specific receptors for heparin (Barzu *et al*, 1984). The molecular weight of the heparin and the source of the endothelial cells are known to influence this process and may have implications for the pharmacokinetics of these compounds (Barzu *et al*, 1984; Barzu *et al*, 1986). Internalisation and the subsequent release of heparin molecules may account for the delayed increase in activity following subcutaneous administration. Heparin bound to the endothelium may be thought to act as an immobilised anticoagulant, but the activity of endothelial-bound heparin is seen to be at least partially neutralised, possibly by endocytosis and intracellular degradation (Barzu *et al*, 1986; Tobelem, 1989). Heparin also binds to platelets, and two binding sites on the molecule have been proposed. Both sites have the ability to bind both platelets and antithrombin, but do so with different preferences (Barrowcliffe & Thomas, 1994). The size of the heparin molecules means that they are multivalent for these sites and therefore have the effect of crosslinking platelets, leading to a fall in the number circulating. Another, more serious form of heparin induced thrombocytopenia (HIT) is due to the development of antibodies against the heparin-platelet complex (Barrowcliffe & Thomas, 1994). Although there have been reports of patients who have developed HIT whilst receiving UFH being successfully switched to low molecular weight heparin (reviewed by Barrowcliffe *et al*, 1992), a more recent investigation did not show any difference between UFH and LMWH in the development of HIT (Amiral *et al*, 1996).

1.3.2.4 Clinical use of unfractionated heparin

UFH is well established as a treatment for venous thrombosis and pulmonary embolism, and has been used for this purpose for over 60 years. It has also been successfully used in the treatment of acute coronary artery syndromes such as unstable angina and myocardial infarction and as an adjunct to thrombolysis. When UFH is infused, it binds to a large number of plasma proteins, endothelial cells and macrophages, which clear it from the circulation in a rapid and saturable manner. The kidneys also remove heparin in a slower, non-saturable manner. This means that the anticoagulant response to therapeutic doses of heparin is non-linear, and laboratory monitoring is required as frequently as every six hours. UFH also has poor bioavailability when injected subcutaneously, probably due to poor absorption of the high molecular weight material, meaning that continuous intravenous infusion is preferred (Bell & Hennebry, 1999; Hirsh *et al*, 2001).

1.3.2.5 Low molecular weight heparin

Low molecular weight heparin may be prepared from the parent compound by separation of the different length chains (eg by gel filtration) or by cleavage of the longer chains (by chemicals such as nitrous acid or enzymatically by heparinase). The mean molecular weight of LMWH preparations is between 3 000 and 6 000, representing chain lengths of 10 to 20 saccharide units, in comparison with UFH preparations of mean molecular weight 15 000, representing around 50 saccharide units (see Figure 1.9). The anticoagulant profile of LMWH preparations will differ depending upon the exact distribution of the chain lengths that they contain. In terms of potencies, all have a higher activity against FXa (anti-Xa activity) than against thrombin (anti-IIa activity) - a preparation with a 10:1 ratio of anti-Xa to anti-IIa

activities will have a high proportion of short polysaccharide chains that are unable to provide the template needed for inhibition of thrombin (Pineo & Hull, 1999).

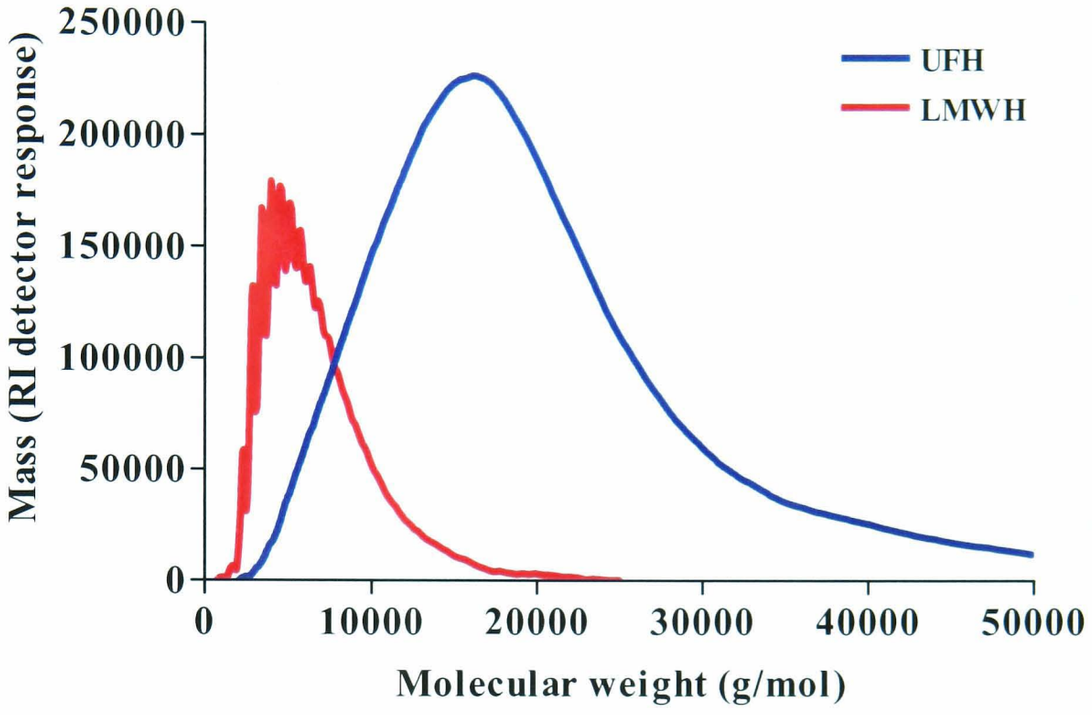


Figure 1.9. *Molecular weight distributions of unfractionated (UFH) and low molecular weight heparin (LMWH).*

UFH was the 4th International Standard (82/502), LMWH was Dalteparin, measured by gel permeation chromatography. Reproduced from Mulloy (2002).

LMWH is now the preferred drug for prevention of venous thrombosis in surgical and medical patients, following trials that showed it to be as safe and effective as UFH. LMWH has also proven to be as effective as UFH in the treatment of acute coronary syndromes and venous thrombosis (Hirsh *et al*, 2001). The clinical advantages of LMWH over UFH are the requirement for only once-daily injection and infrequent monitoring, meaning that in-patient treatment is not required. The improved pharmacokinetics of LMWH are due to the predominance of shorter polysaccharide chains that have less negative charge, and therefore reduced affinity for non-specific binding to proteins, and to the molecular sieving effect of

subcutaneous tissues (Bendetowicz *et al*, 1994; Cosmi *et al*, 1997; see Figure 1.9). This results in increased bioavailability from subcutaneous injection, a longer half-life and more predictable clearance (Dawes *et al*, 1986; Barrowcliffe *et al*, 1992; Pineo & Hull, 1999).

1.3.2.6 Pentasaccharide

In contrast to heparin preparations derived from animal tissue, a synthetic form of the antithrombin-binding pentasaccharide sequence of heparin is now clinically available. Fondaparinux sodium is chemically synthesised to yield a single pentasaccharide molecule with molecular weight of 1 728 Daltons (Da) that potentiates the anti-Xa activity of antithrombin 270-fold but has no appreciable effect on the anti-IIa activity (Olson *et al*, 1992). Clinical trial results show that it may be more effective than pre- or post-operative LMWH in the prevention of venous thrombosis following orthopaedic surgery (Turpie *et al*, 2002; Lassen *et al*, 2002). Advantages of fondaparinux over LMWH include more predictable pharmacokinetics and bioavailability following a once daily subcutaneous injection (Boneu *et al*, 1995), and no cross-reaction with HIT antibodies to the heparin-PF4 complex (Amiral *et al*, 1997), but concerns have been raised over an increased bleeding tendency that was seen in two trials (Bounameaux & Perneger, 2002).

1.3.2.7 Heparin cofactor II

Heparin cofactor II (HC II) is a serpin with molecular weight 66 000. Thrombin is the only procoagulant to be inhibited by HC II and its specificity is due to the presence of a leucine-serine peptide group in its reactive site compared with an arginine-serine group in antithrombin. Thrombin attacks this bond and forms a

stable stoichiometric complex with the inhibitor. Binding to glycosaminoglycans such as heparin, heparan sulphate (HS) and dermatan sulphate (DS) increases the inhibitory action of HC II. The binding of HC II to heparin is of a lower affinity than antithrombin and 10-fold more heparin is required to accelerate thrombin inhibition by HC II. HC II - heparin binding is non-specific, in that it binds to heparin chains longer than 4 saccharides regardless of their composition (Tollefsen, 1995), but greater inhibition of thrombin is achieved with heparin chains at least 20 saccharides long, corresponding to a molecular weight above 8 000. This is longer than the critical chain length for heparin potentiation of antithrombin (18 saccharides and molecular weight above 5 400), probably due to the larger size of the HC II molecule, and is independent of the presence of the specific pentasaccharide sequence (Bray *et al*, 1989).

The N-terminal of HC II resembles the C-terminal of the specific thrombin inhibitor hirudin (see Section 1.3.7.1) and includes a tandem repeat of two highly acidic regions, each containing a sulphated tyrosine residue. The deletion of the first of these repeats leads to a marked reduction in the rate of thrombin inhibition in the presence, but not the absence, of a GAG suggesting that a conformational change may occur following the HC II – GAG interaction that allows the acidic N-terminal region of HC II to bind to the anion binding exosite I of thrombin, in competition with fibrinogen and fibrin. HC II acts independently of exosite II of thrombin, in contrast to antithrombin whose inhibitory action at this site is potentiated by the template mechanism of heparin (Tollefsen, 1997).

The physiological significance of HC II in the inhibition of thrombin appears to be as a back-up to antithrombin, which circulates at 2.5 μM (Murano *et al*, 1980) compared to HC II at 1.2 μM in normal patients (Tollefsen & Pestka, 1985). Increased levels of HC II – thrombin complexes were found in patients with congenital AT deficiency, and in pre- and post-delivery plasma samples from pregnant women (Liu *et al*, 1995).

1.3.2.8 Heparinoids

The term 'heparinoid' may be used to describe all heparin-like compounds, including heparin, but it is commonly used to describe those other than heparin itself such as HS and DS. Heparan sulphate is less highly sulphated than heparin and is found on the surface of a number of cell types, including endothelium. This is in contrast to heparin, which is only synthesised in mast cells where it is stored in secretory granules.

Dermatan sulphate is a naturally-occurring potentiator of the HC II – thrombin interaction and is usually found in the extracellular matrix of the skin and deeper tissues of the vascular wall where it may inhibit extravascular coagulation following injury (Bell & Hennebry, 1999). DS is also expressed in the placenta where it may oppose the hypercoaguable effects of pregnancy, playing a key role in maintaining the patency of the placental microvasculature (Delorme *et al*, 1998) and counteracting the increase in maternal thrombin generation that is observed after delivery (Andersson *et al*, 1996). HC II is the only serpin that is able to bind to DS, but requires molecules of at least six saccharides that contain a specific structure consisting of a triple repeat of a disaccharide, iduronic acid-2-sulphate linked to

N-acetyl galactosamine 4 sulphate. Binding of HC II to this sequence results in a 1 000-fold increase in the affinity of HC II for thrombin (Maimone & Tollefsen, 1990). Clinical trials of DS have shown it to be effective in the prevention of post-operative thrombosis, where it may be at least as effective as UFH with a possible reduction in haemorrhagic side effects (Agnelli *et al*, 1992; Prandoni *et al*, 1992; Di Carlo *et al*, 1999) and these beneficial effects may be due to the ability of DS to inhibit clot-bound thrombin (Bendayan *et al*, 1994).

Danaparoid is a mixture of HS, DS and chondroitin sulphate (CS) that is derived from porcine mucosa following the removal of heparin. One would expect its mode of action to be potentiation of the HC II mediated inhibition of thrombin, but it appears to have higher anti-Xa than anti-IIa activity. Only 55 % of the HS in danaparoid includes the pentasaccharide sequence with high affinity for antithrombin, and the low affinity material is critical to the compound's activity, but its function is not fully understood. Danaparoid is licensed in the USA for use in the prevention of post-operative deep vein thrombosis (DVT), but is frequently used as an alternative treatment for patients who have developed HIT (Acostamadiedo *et al*, 2000).

Pentosan polysulphate is a heparinoid derived from the wood of beech and birch trees, where it is a component of the structural xylans. The extracted xylan is sulphated by chemical treatment and is able to inhibit FXa and thrombin independently of antithrombin or HC II (Fischer *et al*, 1982) but interaction with the serpins may also have an effect, reviewed by Maffrand (1991). Pentosan has been used clinically for the prevention of thrombosis, and its non-animal origin may be

advantageous in light of recent caution regarding transmissible diseases, but negative results in a study of its oral bioavailability may limit its future potential (Faaij *et al*, 1999).

1.3.3 Thrombomodulin and protein C

The activation of coagulation leads to the generation of thrombin, which has potent procoagulant effects but also binds to the endothelial cell membrane protein thrombomodulin (TM), initiating an anticoagulant mechanism. Thrombomodulin binds to the anion binding exosite I of thrombin and a secondary interaction occurs between thrombin and a CS moiety in the TM structure, conferring a higher affinity of TM for thrombin, although there is debate over whether all TM molecules include the CS moiety (Esmon, 1999). In an additional anticoagulant function, the CS moiety may also bind to AT and increase its affinity for thrombin, facilitating the formation of thrombin-antithrombin complexes that then dissociate from TM (Preissner *et al*, 1987). Once bound to TM, thrombin is no longer procoagulant due to the occupation of exosite I by TM, preventing the binding of other substrates such as fibrinogen, FV and FXIII. However, the TM-bound thrombin becomes able to activate Protein C, a zymogen of a serine protease, by cleaving a dodecapeptide from the amino terminal of the heavy chain of Protein C, resulting in the creation of a new amino terminal with leucine as the final residue (Kisiel, 1979). Protein C binds to cell membrane phospholipids in the presence of Ca^{2+}, facilitating its interaction with TM. However, Protein C also binds with increased affinity to its specific receptor on the endothelial cell membrane - the endothelial cell protein C receptor (EPCR) (Fukudome & Esmon, 1994). This binding is mediated via the γ-carboxyglutamic

acid (gla) domain of Protein C and enhances the rate of its activation by TM (Stearns-Kurosawa *et al*, 1996).

Following the activation of Protein C to activated Protein C (APC), and in association with its cofactors Protein S and FV, APC becomes able to inactivate the membrane bound coagulation cofactors FVa and FVIIIa. Endothelial cells have been shown to be a more efficient site of inhibition of FVa compared to activated platelets (Oliver *et al*, 2002). This may be because endothelial cells synthesise and secrete Protein S (Stern *et al*, 1986), which enhances the affinity of APC for the phospholipid membrane surface (Walker, 1981), increases the rate of inactivation of FVa and FVIIIa (Dahlback, 1986; Koedam *et al*, 1988) and prevents the incorporation of these activated cofactors into enzymatic complexes with other clotting factors (Solymoss *et al*, 1988; Regan *et al*, 1994). Interestingly, FV is also secreted by endothelial cells and has been shown to act synergistically with Protein S in increasing the inactivation of FVIIIa (Shen & Dahlback, 1994). The physiological role of the Protein C pathway is likely to be the inhibition of coagulation downstream of the site of vascular damage, where activated coagulation factors need to be scavenged and inactivated to prevent pathological extension of the clot. TM, EPCR, Protein S and FV on undamaged endothelial cells will remove thrombin from the bloodstream and activate PC to inactivate essential coagulation cofactors FVa and FVIIIa, thus preventing coagulation downstream. EPCR, which has the effect of increasing the affinity of PC for thrombin, is primarily found on the endothelium of large vessels and not in capillaries, where thrombotic complications are often found in Protein C deficiency (Laszik *et al*, 1997). TM has a more uniform distribution, and in the capillaries where there is a higher ratio of surface area to blood volume,

the capillary blood is therefore exposed to a sufficient number of the lower affinity inhibitor complexes (TM-thrombin-APC without EPCR) to prevent thrombosis without the risk of haemorrhage (Esmon, 2000).

The interaction of thrombin with TM has been shown additionally to potentiate 1 000-fold the effect of thrombin on the thrombin-activatable fibrinolysis inhibitor (Bajzar *et al*, 1996). Once activated, this enzyme inhbits fibrinolysis by interfering with the cofactor activity of fibrin on plasminogen activation indicating a procoagulant effect of TM, contrary to its other anticoagulant activities. However, this effect would be downstream of the site of coagulation, where thrombin needs to be inhibited to prevent further coagulation whilst any pre-formed fibrin needs to be protected from premature lysis, allowing wound healing to be completed. This suggests a balanced down-regulatory effect on both the coagulation and the fibrinolytic cascades with the aim of focussing the site of coagulation and fibrinolysis.

APC has a longer half-life (15 – 20 minutes) than many serine proteases that are potentiators of coagulation rather than inhibitors and it is slowly inactivated by Protein C inhibitor, α_1-antitrypsin and α_2-macroglobulin (Scully *et al*, 1993; Dahlback & Stenflo, 1994). Deficiencies in Proteins C or S lead to major thrombotic problems, and the most common inherited thrombophilia is due to a single nucleotide polymorphism in the FV gene that makes FVa resistant to inactivation by APC. This mutation is known as Factor V Leiden (De Stefano *et al*, 1998) and indicates the significance of this negative feedback loop and the critical role played by the endothelium in the maintenance of fluidity of the blood. In addition, homozygotes

for the R2 haplotype of FV, which is a series of linked polymorphisms, have a thrombotic tendency resulting from lower plasma concentrations of FV and lower cofactor activity in APC-catalysed inhibition of FVIIIa (Hoekema *et al*, 2001). Deficiency of Protein S also leads to thrombophilia, indicating that its cofactor activity to Protein C has an important role in the inhibition of coagulation (Gandrille *et al*, 1997). Deletion of the thrombomodulin gene has been shown to be lethal in mice, due to a developmental function of TM (Healy *et al*, 1995), but targeted mutation of the TM gene has led to the production of viable mice with a prothrombotic tendency towards fibrin deposition in certain tissues (Weiler-Guettler *et al*, 1998).

The antithrombotic functions of TM and Protein C make them ideal candidates for use as antithrombotic drugs. Recombinant soluble thrombomodulin (r-TM) is able to inhibit clot-bound thrombin and has an antithrombotic effect in animal models of endotoxin-induced disseminated intravascular coagulation (DIC), a coagulopathy where clotting factors and their inhibitors are rapidly consumed (Maruyama, 1999). Safety trials have been completed in humans and the results of phase II clinical trials are awaited. Both Protein C and APC have been used in the treatment of DIC resulting from bacterial sepsis, where endogenous plasma levels are seen to fall rapidly, and in addition to their anticoagulant effects Protein C and APC inhibit the endotoxin-induced nuclear translocation of nuclear factor kappa B (NFκB), the first stage of many inflammatory responses (White *et al*, 2000). A possible limitation of this effect is the downregulation of TM and EPCR following stimulation with inflammatory cytokines (Esmon, 2001), but nonetheless the PROWESS phase III

clinical trial showed a significant decrease in mortality in patients with severe sepsis treated with APC (Bernard *et al*, 2001).

1.3.4 Tissue factor pathway inhibitor

Tissue factor pathway inhibitor (TFPI) is a 276 amino acid protein synthesized primarily by the microvascular endothelium, but also by megakaryocytes. Endothelial cells constitutively express TFPI and the level of expression by endothelial cells and other cell types, such as monocytes, may increase following stimulation. The majority of TFPI (80 - 85 %) is found on the luminal surface of endothelial cells, where its highly basic C-terminal binds to cell surface HSPGs, in addition to non-specific binding elsewhere on the molecule (Iversen *et al*, 1996). Full length TFPI is released from the endothelium following the administration of heparin and other anionic molecules, independent of the presence of an antithrombin binding sequence on the molecule (Cella *et al*, 2001). This effect is dependent on the molecular weight of the heparin, with UFH releasing more full-length, free TFPI from the endothelium than LMWH (Alban & Gastpar, 2001; Brown & Kuter, 2001). The release of TFPI from the endothelium contributes to the antithrombotic effect of heparin, which has also been shown to act synergistically with TFPI (Wun, 1992; Bregengaard *et al*, 1993). The interaction of TFPI with heparin is charge dependent, with the high density of negative charge on UFH allowing it to bind with higher affinity to the basic C-terminal of TFPI than LMWH (Valentin *et al*, 1994). Around 10 % of the total TFPI circulates in the plasma associated with lipoproteins in a less active C-terminal truncated form, and the remainder is found in platelets (Bajaj *et al*, 2001). The level of circulating TFPI is higher during pregnancy and lower in the neonate and the level of mRNA expression is at its highest in the placenta and its

lowest in the brain. This suggests that a compensatory mechanism is in operation, countering the relatively thrombophilic condition of pregnancy but not compromising haemostasis where bleeding would cause major problems (Bajaj *et al*, 1999).

As its name suggests, TFPI inhibits one of the major complexes in the extrinsic coagulation pathway. TFPI has three Kunitz type protease inhibitory domains, and the first stage of its inhibitory action is to bind to and inhibit FXa via the Kunitz 2 domain. This binary complex then binds to FVIIa in the TF-FVIIa complex on the cell surface, via the Kunitz 1 domain on TFPI, to form a quaternary complex. The rapid formation of the quaternary complex may be slowed down by the presence of FVIII and FIX, in a mechanism that may allow the extrinsic pathway of coagulation to become established and the absence of which leads to bleeding in haemophiliacs, where there is increased reliance on the extrinsic pathway (Broze, 1994; Bombeli *et al*, 1997; Verstraete, 1998). The dampening effect of TFPI in an intact coagulation system, by inhibiting the actions of FX and FXa, serves to prolong the lag time of thrombin generation where feedback activation of coagulation factors by trace amounts of thrombin would amplify the response, and to reduce the rate of thrombin generation once the burst phase has been reached (van't Veer & Mann, 1997).

TFPI-2 is a structural homologue of TFPI that has been found to have similar activity against the TF-FVIIa complex and FXa and to have its activity potentiated by heparin (Sprecher *et al*, 1994). TFPI-2 is expressed in the placenta, where it has previously been identified as placental protein 5 (PP5) or matrix associated serine

protease inhibitor (MSPI) and is presumed to have a role in maintaining the blood flow through the TF rich villi (Udagawa *et al*, 2002).

1.3.5 Protease nexin 1 and 2

Endothelial cells synthesise protease nexin 1 (PN-1; Eaton & Baker, 1983; Leroy-Viard *et al*, 1989), a serpin that is structurally homologous to AT (Sommer *et al*, 1987) and C-1 inhibitor (Van Nostrand *et al*, 1988). PN-1 forms a stoichiometric complex with thrombin, FXa, FXIa and trypsin at a rate that is markedly accelerated by heparin, regardless of the presence of the antithrombin-specific pentasaccharide sequence (Evans *et al*, 1991; Knauer *et al*, 2000). In solution, PN-1 is also able to inhibit urokinase and plasmin, but loses this ability when bound to the cell surface or ECM, (Wagner *et al*, 1989) possibly due to interaction with a cofactor suggested to be collagen type IV (Donovan *et al*, 1994). Surface-bound PN-1 remains able to bind thrombin and FXIa and the PN-1 – protease complex is internalised by the cell and degraded (Knauer *et al*, 1983; Knauer *et al*, 2000). The increased affinity of cell-bound PN-1 as a result of its interaction with cell surface HSPG and its altered specificity when bound to ECM suggests that PN-1 is important in the inhibition of cell surface or extravascular coagulation.

Protease nexin 2 (PN-2) is the secreted form of the amyloid β-protein precursor (APP), implicated in senile plaques in diseases such as Alzheimer's disease. It is predominantly found in the α-granules of platelets (Van Nostrand *et al*, 1990) and its mRNA may be detected in other cell types, including endothelial cells (Van Nostrand *et al*, 1991). PN-2 inhibits FXIa (Smith *et al*, 1990), FXa (Mahdi *et al*, 1995) and is a more potent inhibitor of FIXa than antithrombin (Schmaier *et al*, 1993). Heparin

potentiates the inhibitory effect of PN-2 on FXIa (Van Nostrand *et al*, 1990) and FIXa (Schmaier *et al*, 1993). PN-2 is able to inhibit FIXa in its function as part of the intrinsic tenase complex on platelets and endothelial cells (Schmaier *et al*, 1995), to inhibit FXa in the prothrombinase complex (Mahdi *et al*, 1995), and also to inhibit FVIIa in the extrinsic tenase complex with TF (Mahdi *et al*, 2000).

1.3.6 Protein Z-dependent protease inhibitor

The protein Z dependent protease inhibitor (PZI) is a 72 000 Da serine protease inhibitor whose activity against FXa is increased 1 000 fold by binding, in the presence of phospholipids and calcium, to the cofactor Protein Z (PZ), a 62 000 Da, vitamin K dependent plasma protein. PZI is also able to inhibit FXIa regardless of the presence of PZ, phospholipids or calcium. Interaction with either FXIa or FXa, following intrinsic or extrinsic activation respectively, leads to proteolytic cleavage and inactivation of PZI in what may be a regulatory mechanism that dampens the coagulation response in areas of minor vascular damage, but allows coagulation to proceed where the stimulation is greater. The clinical implications of PZI or PZ deficiency remain to be established (Broze, 2001).

1.3.7 Other antithrombotic drugs

In addition to the naturally occurring inhibitors of coagulation mentioned above, numerous exogenous agents have been developed for use as antithrombotic agents in clinical situations where drugs such as heparin may have limitations, for instance an inability to inhibit clot-bound thrombin. Many small molecule inhibitors of coagulation proteases have been developed and trialled in a number of clinical

situations, and five direct thrombin inhibitors have recently proven to be safe and effective alternatives to heparin in acute coronary syndromes (The Direct Thrombin Inhibitor Trialists' Collaborative Group, 2002).

1.3.7.1 Hirudin

Hirudin is a potent and selective inhibitor of thrombin that was originally isolated from the saliva of the medicinal leech *(Hirudo medicinalis)*. It is a 65 amino acid protein, with three disulphide bonds and a molecular weight of around 7 000, which forms a slowly reversible, stoichiometric complex with thrombin through an electrophilic interaction. It binds to both the fibrinogen-binding site and the catalytic site of thrombin, thereby preventing the conversion of fibrinogen to fibrin and disrupting the positive feedback effects of thrombin on coagulation (Badimon *et al*, 1991). It is commonly produced in a recombinant form missing a sulphate residue on tyrosine 63 (and therefore known as desulfato hirudin), with no loss of activity (Lauer & Lincoff, 1999; Prisco *et al*, 2001). Other analogues of hirudin with lower affinities for thrombin have been manufactured with the aim of reducing the potential side effects of such a potent inhibitor. Hirugen is a 12 amino acid peptide, based on the C terminal fragment of hirudin, which binds to the exosite of thrombin thus interfering with substrate recognition (Verstraete *et al*, 1998; Lauer & Lincoff, 1999). Hirulog is the brandname for bivalirudin, a 20 amino acid chimeric molecule made by linking the C terminal and the N terminal of hirudin with a sequence of four glycines. Clinical trials using hirulog for the prevention of DVT in orthopaedic patients, in coronary angioplasty and as an adjunct to thrombolytic therapy have all given encouraging results (Verstraete *et al*, 1998; Kimball, 1999). Hirunorms are

peptide analogues of bivalirudin that are designed to resist proteolytic cleavage for longer and to be more specific for thrombin (Verstraete *et al*, 1998).

Hirudin has been found to have beneficial effects when compared with heparin in the prevention and treatment of DVT following surgery (Harker *et al*, 1997; Prisco *et al*, 2001) and is indicated as an antithrombotic agent in patients with HIT, where an immune reaction leads to the elimination of platelets (Prisco *et al*, 2001). The ability of hirudin to inhibit clot-bound thrombin may also prevent the growth of existing clots in vessels that have been reperfused following thrombolytic therapy, where thrombin may become re-exposed following the dissolution of layers of a clot (Harker *et al*, 1997). Hirudin has an advantage over heparin in this situation as the heparin-antithrombin complex is unable to inhibit clot-bound thrombin because the binding site for heparin (thrombin exosite II) is inaccessible (Weitz *et al*, 1990). Another advantage of hirudin over heparin is that the direct action of hirudin means that there is no dependence on the availability of plasma cofactors such as antithrombin, which may be congenitally absent or depleted due to long-term heparin therapy (Lauer & Lincoff, 1999). The drawback may be bleeding complications with no direct antidote (Prisco *et al*, 2001).

1.3.7.2 Other thrombin inhibitors

Other reversible inhibitors of thrombin include argatroban, napsagatran, inogatran and efegatran. Argatroban is a direct thrombin inhibitor based on the amino acid L-arginine. It binds reversibly to the catalytic site of thrombin and has been shown to be an effective antithrombotic in venous and arterial models of thrombosis. Clinical trials suggest that argatroban is effective as an adjunct to thrombolytic

therapy (Kimball, 1999) and is beneficial as an alternative to heparin therapy in patients with HIT (Lewis *et al*, 2001) although there is concern that a rebound prothrombotic state may occur when the therapy is stopped and active thrombin dissociates from the inhibitor (Kimball, 1999). Napsagatran is a selective and reversible 559 Da inhibitor of the active site of thrombin that delays thrombin generation more effectively than hirudin, but less effectively than UFH (Kimball, 1999; Bounameaux *et al*, 1999). Unlike hirudin, it has higher affinity for clot-bound thrombin than for fluid phase thrombin, suggesting a particular suitability for treatment of arterial thrombosis or thrombolysis (Gast *et al*, 1994). Inogatran is a synthetic dipeptide of 439 Da that is another competitive and reversible inhibitor of thrombin. It performed no better than heparin in trials and clinical development has ceased (Verstraete *et al*, 1998; Kimball, 1999). Efegatran, a tripeptide aldehyde is also a reversible inhibitor of the anion-binding site of thrombin but it has poor selectivity for thrombin and also binds to FXa and other serine proteases. Clinical trials using this inhibitor as an adjunct to thrombolysis have shown no benefit over current therapy (Fung *et al*, 1999; The PRIME Investigators, 2002).

Inhibitors that bind covalently to thrombin include CVS-1123 and DuP714. CVS-1123 is a 575 Da peptide that binds slowly and tightly to thrombin and its bioavailability profile suggests that it has potential as an oral antithrombotic drug, although no information is available regarding clinical studies (Verstraete *et al*, 1998; Kimball, 1999). DuP714 is a potent active site inhibitor of thrombin that may be orally active, but may cause problems by inhibiting plasmin leading to impaired fibrinolysis, and has an undesirable kinetic profile that shows slow, tight binding (Verstraete *et al*, 1998; Kimball, 1999).

Ximelegatran is an oral direct thrombin inhibitor that is metabolised to the active form melagatran which has been shown to reduce thrombin generation in blood shed from bleeding time wounds (Sarich *et al*, 2002). The first clinical study of oral ximelegatran, subcutaneous melagatran or LMWH showed it be the equal of LMWH in the prevention of venous thromboembolism following orthopaedic surgery (Eriksson *et al*, 2002). This is an encouraging result in the search for an oral antithrombotic agent to replace the widespread use of the oral anticoagulant warfarin and the non-specific anti-inflammatory drug, aspirin.

1.3.7.3 Factor X inhibitors

Factor X can be activated by the intrinsic tenase complex or the extrinsic complex of TF-FVIIa and is the beginning of the common pathway of coagulation, making it an attractive target for direct inhibition. The involvement of FXa in the prothrombinase complex means that its inhibition will prevent the amplification of this stage of the coagulation cascade in the generation of thrombin, and this may be a more effective strategy than attempting to neutralise pre-existing thrombin and all its positive feedback effects.

As previously mentioned (Section 1.3.2.6), a synthetic form of the pentasaccharide sequence of heparin is clinically available and is used to potentiate the anti-Xa activity of endogenous antithrombin. In addition, a number of naturally occurring direct inhibitors of FXa have been isolated and characterised. The tick anticoagulant protein (TAP), isolated from the soft tick *(Ornithodorus mubata)*, and its recombinant form (rTAP) are 60 amino acid, 6 850 Da monomeric peptides that bind slowly to FXa in a two-step mechanism to form a tightly bound complex. It has a

much higher affinity for FXa assembled into the prothrombinase complex, and has been shown to be an effective antithrombotic agent in animal models of venous and arterial thrombosis, reocclusion and restenosis (Samama *et al*, 1998; Kunitada *et al*, 1999). Antistasin, a 119 amino acid, 17 000 Da polypeptide isolated from the Mexican leech *(Haementeria officinalis)* also binds slowly and tightly to FXa in a two-step mechanism. It was able to inhibit extrinsic coagulation in an animal model of venous thrombosis (Vlasuk *et al*, 1991) but has proven to be antigenic and therefore of limited clinical potential (Samama *et al*, 1998; Kunitada *et al*, 1999). The hookworm *Ancylostoma caninum* has also been found to produce a family of anticoagulant proteins (AcAP; or nematode anticoagulant peptides, NAP). The recombinant form of nematode anticoagulant peptide 5 (rNAP-5) is an 8 700 Dalton peptide inhibitor of FXa that has been shown to inhibit arterial thrombosis in an animal model (Kunitada *et al*, 1999) and NAP-6 has similar inhibitory activity (Stanssens *et al*, 1996). Another member of this family of peptides is rNAPc2, an 85 amino acid peptide serine protease inhibitor, which has been shown to inhibit the TF-FVIIa complex following its initial binding to FX or FXa (Stanssens *et al*, 1996), and has been shown to be effective in the prevention of DVT following orthopaedic surgery (Lee *et al*, 2001).

The main function of the 43 000 Dalton endogenous protein TFPI is the inhibition of the TF-FVIIa complex, as discussed in Section 1.3.4, but it is also reported to have a direct inhibitory effect on FXa (Prager *et al*, 1995). A clinical trial has shown treatment with TFPI (compared to placebo) to reduce mortality in the treatment of patients with sepsis (Abraham *et al*, 2001), and the results of further trials are awaited.

A non-peptide derivative of propanoic acid, known as DX-9065a, is a rapid-binding, specific, direct competitive inhibitor of FXa. It is able to inhibit both fluid phase FXa and FXa that has been incorporated into the prothrombinase complex, and it inhibits the intrinsic generation of thrombin more effectively than the extrinsic. As it competes for the binding of FXa with the other components of the prothrombinase and the TF-FVIIa complexes, sufficient thrombin is generated to activate platelets at sites of vascular damage, although fibrin formation does not occur, and this may prevent excessive bleeding whilst preventing thrombosis (Samama *et al*, 1998; Kunitada *et al*, 1999). Initial clinical results show that the drug is well tolerated and has a promising pharmacokinetic profile worthy of further study (Dyke *et al*, 2002).

1.3.7.4 Anti-platelet agents

Platelets play a major role in haemostasis and are therefore ideal targets for inhibitors of coagulation. The best known and most widely used platelet inhibitor is aspirin, which irreversibly inactivates platelet cyclooxygenase, preventing the conversion of arachidonic acid to endoperoxides and thromboxane A_2. The potentiating effect that thromboxane A_2 has on the action of other agonists is therefore lost, effectively blocking the activation of the platelet (Bennett, 2001). Unfortunately, the inhibition of cyclooxygenase is not confined to platelets, and unwanted effects such as gastric bleeding and an increased bleeding time preclude some patients using it (Scrip, 1998; FitzGerald & Patrono, 1998). For this reason, there has been much interest in the development of a specific, preferably oral, inhibitor of platelet function that does not have bleeding side effects. Antagonism of the $P2Y_{12}$ receptor for adenosine diphosphate (ADP), a potent activator of platelets, has been the subject of high-profile clinical trials. Metabolites of oral drugs such as clopidogrel and ticlopidine

are able to irreversibly modify the structure of the receptor, rendering it insensitive to ADP. The benefits of clopidogrel over aspirin in certain indications studied in the CAPRIE trial have been reported (Bennett, 2001).

An alternative approach to the inhibition of platelet-mediated thrombosis is the inhibition of aggregation and many drugs targeted at inhibition of the main aggregatory receptor GP IIb/IIIa are in development (Topol *et al*, 1999). Perhaps the best-known of these drugs is abciximab (c7E3, ReoPro™), which is a fragment of a humanised monoclonal antibody against this receptor. Abciximab has been studied extensively in a number of clinical situations and is now licensed in the US for use in acute coronary care (Califf *et al*, 1995; EPILOG Investigators, 1997; CAPTURE Investigators, 1997; Swainger, 1997; Scrip, 1998; FitzGerald & Patrono, 1998; Bennett, 2001). In addition to the anti-aggregatory effect of the drug, it has been found to inhibit *in vitro* platelet-dependent generation of thrombin – in other words, it also acts as an anticoagulant (Reverter *et al*, 1996). This work has been repeated with peptide and non-peptide antagonists of the receptor (Pedicord *et al*, 1998; Herault *et al*, 1998) and the mode of action shown to be an inhibition of the exposure of negatively charged phospholipids on the outer leaflet of the membrane of activated platelets (Pedicord *et al*, 1998). Abciximab is also known to cross-react with the vitronectin receptor on platelets and other cells (Reverter *et al*, 1996), and with the Mac-1 receptor on monocytes, interfering with monocyte adhesion to fibrinogen and ICAM-1. This indicates an inhibitory effect on cell adhesion to the subendothelium (Simon *et al*, 1997).

At present there are no licensed products that inhibit adhesion, the first stage of platelet-mediated thrombosis. Primate models of arterial thrombosis have demonstrated inhibitory effects of antibodies against GP Ibα (in combination with an anti-GP IIb/IIIa antibody; Wu *et al*, 2002a) and vWF (Wu *et al*, 2002b), suggesting that this is a potential target for antithrombotic therapy. Polymorphisms in the collagen receptors of platelets are currently being studied in the hope that inhibitory agents may be developed that inhibit collagen-mediated platelet adhesion and activation (Kunicki, 2002).

1.1.1 Endothelial cell effects on platelets

Agents secreted by endothelial cells also have effects on platelets. Prostacyclin and nitric oxide (NO) are released from endothelial cells in response to agonists such as thrombin, ATP, ADP, bradykinin and histamine, most of which are secreted from or associated with activated platelets. Endothelial cells are a major source of prostacyclin, and its synthesis and secretion is rapidly upregulated following stimuli that lead to an increase in intracellular calcium levels. This leads to the phosphorylation of the extracellular-signal regulated protein kinase (ERK) that then phosphorylates phospholipase A_2, which mediates the first step in the prostanoid synthetic pathway (Wheeler-Jones *et al*, 1996). Prostacyclin synthesis tends to be brief due to the burst of calcium required from intracellular stores. Prostacyclin stimulates adenylate cyclase in platelets, therefore increasing cAMP levels and inhibiting platelet activation (Pearson, 1999). Deficiency of the prostacyclin synthase enzyme has been found to be prothrombotic in humans (Nakayama *et al*, 2002).

Nitric oxide is expressed by endothelial cells following its synthesis by the calcium-dependent nitric oxide synthase (NOS) in response to a similar range of agonists to that which stimulates prostacyclin release. The most important physiological regulator is shear stress, discussed in Section 1.4.3. Increased intracellular calcium concentrations stimulate the action of NOS in generating NO from arginine. This requires a lower level of calcium than prostacyclin synthesis and may therefore be maintained for a longer period as intracellular calcium stores do not become exhausted. NO acts by elevating guanylate cyclase activity leading to increased intracellular levels of cGMP that in turn inhibit calcium influx into the platelet, thus inhibiting its activation (Smith *et al*, 1994). Endothelial-derived NO may not have a significant effect on platelets due to its brief half-life, but knockout mice lacking NOS in platelets and endothelial cells have decreased bleeding times, indicating a loss of inhibition (Freedman *et al*, 1999) and NO released from endothelial cells effectively inhibits platelet adhesion to the endothelium (Radomski *et al*, 1987).

This negative feedback loop, where agonists released from activated platelets cause endothelial cells to release inhibitors that prevent further platelets becoming activated, may act as a dampening mechanism to inhibit the coagulation or inflammation response to low levels of stimulation.

1.1.2 Procoagulant activity of endothelial cells

Procoagulant tissue factor is synthesised by endothelial cells and although it is not constitutively expressed at the cell surface, it is expressed in response to stimulation by a number of factors. Thrombin activates endothelial cells by its interaction with a family of G-protein linked protease activated receptors (PAR), which have part of the

N terminus cleaved by the enzyme to reveal a new sequence that then acts as a tethered ligand for the receptor (Vu *et al*, 1991). The main thrombin receptor on endothelial cells and platelets is PAR-1, but endothelial cells also express PAR-2 (Molino *et al*, 1997) and PAR-3 (Schmidt *et al*, 1998). Endothelial cells also express tissue factor following their exposure to endotoxin (LPS; Lyberg *et al*, 1983; Brox *et al*, 1984) tumour necrosis factor (TNF; Bevilacqua *et al*, 1984), and interleukin-1 (IL-1; Bevilacqua *et al*, 1986). This is particularly important in bacterial sepsis, where interaction between the coagulation and inflammation systems can lead to DIC, multiple organ failure and high rates of mortality (Esmon, 2001). In addition to the expression of tissue factor, thrombin activated endothelial cells express platelet-activating factor (PAF), a phospholipid that is involved in the adhesion of polymorphonuclear leukocytes to endothelial cells in the early stage of inflammation (Zimmerman *et al*, 1990).

In addition to their possible role in activation of the intrinsic pathway of coagulation, discussed in Section 1.3.1, endothelial cells may promote coagulation in a number of ways. A specific receptor for FIX and FIXa has been identified on the endothelial cell membrane (Rimon *et al*, 1987) and the binding affinity of FIXa increases in the presence of FVIII and FX (Stern *et al*, 1985), enhancing procoagulant activity. Blocking the active site of FIXa prevents the generation of FXa in the presence of TF-expressing TNF-activated endothelial cells or their TF-containing extracellular matrix, indicating a cooperative role for the intrinsic and extrinsic tenase complexes (Tijburg *et al*, 1991). Specific binding sites for FXa have been identified on endothelial cells (Bono *et al*, 1997) that can facilitate the activation of FVII (Rao *et al*, 1988); and the synthesis, expression and binding of FV and FVa (Maruyama *et al*,

1984; Cerveny *et al*, 1984) can enhance the formation of the prothrombinase complex on the cell surface.

Endothelial cells are the source of at least 90 % of circulating vWF (Bowie *et al*, 1986), a protein that acts as a stabilising protein for circulating FVIII and as a ligand for the adhesion of platelets to components of the extracellular matrix, as discussed earlier. As well as constitutively secreting a dimeric form of vWF from their cytosol, endothelial cells release their stores of multimeric vWF from Weibel-Palade bodies following stimulation with thrombin (Levine *et al*, 1982). The release of stored vWF accompanies a transient expression of P-selectin on the cell membrane, as the granule membrane fuses and is exposed. It is unclear whether the increased circulating vWF levels may be implicated in prothrombotic tendencies during infection or are just a marker of endothelial activation (Pearson, 1999).

1.1.3 Fibrinolytic activity of endothelial cells

Endothelial cells also play a role in the initiation of fibrinolysis following clot formation, primarily by the synthesis of tissue-type plasminogen activator (t-PA). In addition to constitutive secretion, agonists such as thrombin stimulate the release of t-PA from storage sites in endothelial cells (Emeis *et al*, 1997). The function of t-PA is to cleave plasminogen to the active plasmin, the major fibrinolytic protein, and plasminogen and t-PA both bind to the surface of endothelial cells, thus localising plasmin generation and therefore fibrinolysis to the cell surface (Hajjar, 1995).

Plasminogen activator inhibitor 1 (PAI-1) is the major inhibitor of t-PA and is also secreted primarily by endothelial cells. Under resting conditions the plasma

concentration of PAI-1 is 400 pM compared to 80 pM for t-PA, resulting in complex formation and rapid clearance from the plasma (Booth, 1999). Inflammatory agonists such as LPS, TNF-α and IL-1 lead to an increase in the level of PAI-1 secretion but not in t-PA thus prolonging the life of the clot (Schleef *et al*, 1988). Platelet α-granules are also rich in PAI-1 which is released on activation, inhibiting fibrinolysis in the early stages of clot formation (Booth, 1999).

1.2 Flow

In vivo the blood is rarely stationary and the constant movement of the circulation has effects on the components of blood and the vessels through which it flows. These effects range from the mechanical effects of the stretching of the vessel and the flow of blood across the luminal surface, to the physical effects of the interaction of fluid phase cells and proteins with the luminal surface, and the biochemical effects of the interactions of these cells and proteins. Many studies have been undertaken *in vivo, ex vivo* and *in vitro* to attempt to understand the isolated or combined effects of these processes. Details on how these conditions have been modelled in the laboratory and explanations of the terminology used can be found in Chapter 3.

1.2.1 Flow and coagulation

The influence of flow is particularly relevant in studies of blood coagulation, as certain circulating clotting factors need to adhere to phospholipid surfaces before they can interact, for example the tenase and prothrombinase complexes. The phospholipid surfaces involved here are usually provided by activated platelets that have adhered to, and spread upon, areas of damaged vessel wall. The 'two-

dimensional' model of blood coagulation on a surface has been elegantly explained by Nemerson (1995). Briefly, when an enzyme, for example the prothrombinase complex, is attached to the wall of a vessel in static conditions, the delivery of the substrate, in this case prothrombin, is achieved by diffusion. Interactions will occur between enzyme and substrate, leading to a zone of substrate depletion near the vessel wall and also a zone of product accumulation. If the enzyme is 100 % efficient at converting every molecule of substrate that it encounters, then the rate limiting step will be the diffusion of the substrate to the enzyme – this is termed the 'transport rate limit'. If, however, the medium is stirred or flowing, the limit is shifted, and the flux of substrate to the enzyme is increased in proportion to the one-third power of the wall shear rate.

This model of coagulation under flow conditions was the result of numerous studies by Nemerson and colleagues on the activation of FX that was perfused with FVII through glass capillaries coated with phospholipids and TF. It was found that FVIIa bound tightly to TF on the vessel wall and this complex converted FX to FXa at a rate that was limited by the rate of substrate delivery, which in turn was a function of the substrate concentration and the wall shear rate (Gemmell *et al*, 1988; Gemmell *et al*, 1991; Andree *et al*, 1994; Andree & Nemerson, 1995; Gentry *et al*, 1995). Studies on the assembly of the prothrombinase complex on phospholipid vessel wall surfaces have reported similar results, with factor FVa inserted into a phospholipid bilayer combining with FXa in the perfusate and then activating prothrombin as it is delivered to the vessel wall, at a rate limited by substrate concentration and wall shear rate (Schoen *et al*, 1990; Billy *et al*, 1995a).

The kinetics of the inhibitors of coagulation in flow conditions have also been studied. Studies on the inhibition of FX activation by TFPI, and the anti-thrombin activity of surface bound heparin have agreed with the theories on transport of reactants to and from the boundary layer (Lindhout *et al*, 1992; Lindhout *et al*, 1995). The consumption of antithrombin in the boundary layer has been shown to limit the effectiveness of unfractionated and pentasaccharide heparin, suggesting that their main inhibitory action is prevention of the activation of the components of the prothrombinase complex (FXa and FVa) in the fluid phase rather than inhibition of the surface-bound complex itself (Schoen & Lindhout, 1991). Confirming this, it has also been shown that prothrombinase bound to lipid membranes is protected from inactivation due to competition between antithrombin and prothrombin for the active site of FXa (Billy *et al*, 1995b).

1.2.2 Flow and platelets

Flow conditions are known to have profound effects on platelets, which must be able to perform their haemostatic function under a wide variety of conditions. As discussed in Section 1.2.1, platelets have a wide range of adhesive receptors and have the specific ability to adhere to thrombogenic surfaces under conditions of high shear stress and are therefore able to mediate haemostasis in damaged blood vessels.

Shear induced platelet aggregation (SIPA) is a recognised phenomenon although the precise mechanism is not understood. It has been hypothesized that the receptors for vWF (probably GP Ib/IX/V rather than GP IIb/IIIa) on the platelet surface are somehow affected by conditions of high shear and become able to bind to vWF, allowing a microaggregate to form, where agents such as ADP and adrenaline

released from activated platelets may become concentrated leading to a positive feedback activation of platelets and the development of a full aggregate. The collision frequency and collision efficiency (the number of collisions that lead to aggregation) of platelets is directly proportional to the shear rate, indicating the importance of shear in primary haemostasis (Kroll *et al*, 1996).

As discussed in Section 1.2.2, activated platelets facilitate coagulation by increasing the local concentration of clotting factors at their cell membrane - platelets carry certain clotting factors and following activation the expression of negatively-charged phospholipids on their outer membrane provides a catalytic surface for coagulation.

1.2.3 Flow and the endothelium

Endothelial cells are constantly exposed to shear stress *in vivo* and there have been numerous studies on the effects of shear on the structure and function of the endothelium. The endothelium's constitutive expression of a non-thrombogenic surface and secretion of agents that inhibit platelet aggregation may be affected by the increased shear rate that can occur when the vessel lumen has narrowed as a result of atherosclerosis or neointimal hyperplasia.

Shear stress affects the morphology and orientation of endothelial cells. The cytoskeleton has been shown to be reorganised over a period of 24 hours in order to streamline the cells and to align them with the direction of the flow (Dewey *et al*, 1981; Levesque & Nerem, 1989; Levesque *et al*, 1989; Barbee *et al*, 1994). These cytoskeletal changes have also been shown to affect the clustering of endothelial cell receptors for ECM proteins and reorganisation of the ECM under endothelial cells

also occurs, with increased levels of adhesive proteins, thickening of fibrils of collagen, laminin and fibronectin, and fibril alignment with flow occurring within hours (Thoumine *et al*, 1995a; Thoumine *et al*, 1995b). Endothelial cell proliferation and migration have been shown to increase following exposure to shear stress in a disturbed flow model, suggesting that remodelling occurs at stenoses or branches in the vasculature (Tardy *et al*, 1997).

Flow can alter the non-thrombogenic characteristics of the endothelium by affecting the expression and secretion of a number of factors. Endothelial cells exposed to steady or pulsatile flow have been shown to dramatically and immediately increase their secretion of the vasodilators and platelet inhibitors prostacyclin and nitric oxide (Frangos *et al*, 1985; Kanai *et al*, 1995). The prolonged increase in NO synthesis in response to shear stress has been demonstrated without a detectable increase in intracellular calcium levels, which is surprising as NOS is a calcium-dependent enzyme. This may be explained by changes in the phosphorylation pattern of NOS in response to stimulation, leading to an increased affinity for calcium such that intracellular stores are sufficient for the response. Shear stress activates protein kinase B (also known as Akt) to phosphorylate Serine^{1177} of NOS, facilitating activation of the enzyme by calmodulin (Dimmeler *et al*, 1999; Fulton *et al*, 1999). Soluble agonists bradykinin and histamine can activate calmodulin-dependent kinase II (CaMKII) to phosphorylate Serine^{1177}, and also stimulate a phosphatase to dephosphorylate Threonine^{495}, further facilitating calmodulin binding to NOS and stimulating production of NO (Fleming *et al*, 2001).

Synthesis and release of the coagulation inhibitor TFPI have also been reported to increase in an endothelial cell line that was subjected to shear stress (Westmuckett *et al*, 2000). The fibrinolytic activity of endothelial cells is also affected by shear stress, with an increase in the secretion of tissue-type plasminogen activator (tPA) being observed in endothelial cells subjected to arterial shear stresses for more than six hours (Diamond *et al*, 1989). These responses indicate that endothelial cells have a protective response to increased shear stress, whereby they act to limit the impact of the shear stress on the other components of the vasculature.

Endothelial cells can also regulate the expression of adhesion molecules on their cell membrane in response to shear stress and the signalling pathways involved in communicating shear stress to the nucleus of endothelial cells are reported to include ion channels, G proteins, focal contact-associated proteins, protein kinases and transcription factors (Papadaki & Eskin, 1997) and caveolae at the cell surface have been identified as specific organelles that may act to convert mechanical stress into chemical signals within the endothelial cell (Rizzo *et al*, 1998). The effect of increased shear has been shown to lead to the transient up-regulation of ICAM-1 and down regulation of vascular cell adhesion factor 1 (VCAM-1), receptors which are implicated in the adhesion and infiltration of leukocytes in the development of atherosclerosis. These effects were mediated at the mRNA level, indicating an effect on gene transcription (Sampath *et al*, 1995), and the reason for the different response may be the presence of a shear stress response element (SSRE) in the promoter for the gene for ICAM-1 but not VCAM-1 (Nagel *et al*, 1994). This is also the case for platelet derived growth factor B (PDGF-B), the gene for which has a SSRE that interacts with NFκB, a DNA binding protein, leading to increased transcription

following exposure to shear stress (Khachigian *et al*, 1995; Resnick & Gimbrone, 1995; Resnick *et al*, 1997). The ability of endothelial cells to down-regulate receptors implicated in atherosclerosis as a response to increased shear, as is found in partially occluded vessels, suggests the existence of a self-regulating defence mechanism. Conversely, an increase in the rate of uptake of low density lipoproteins (LDL) by pinocytosis, implicated in the formation of atherosclerotic plaques, may occur (Davies *et al*, 1984; Levesque *et al*, 1989) and endothelial cells exposed to shear stress *in vitro* release increased amounts of vWF, which may contribute to platelet adhesion and coagulation (Galbusera *et al*, 1997).

Steady laminar flow is considered to produce the optimal antithrombotic state in endothelial cells and it has been demonstrated that oscillatory or disturbed flow conditions, as may be found at bifurcations in the vasculature, can lead to a prothrombotic state and altered gene expression (Garcia-Cardena *et al*, 2001). Upregulation of proatherosclerotic genes in individual endothelial cells may provide the initial focus for atherosclerosis at these high-risk locations (Davies *et al*, 2001). Examples of potential triggers include i) the increased transcription and expression of procoagulant TF in disturbed flow in comparison to the upregulation of the inhibitor TFPI in the presence of unidirectional flow (Mazzolai *et al*, 2001); ii) a relative increase in the oxidative stress of the endothelium (Silacci *et al*, 2001); iii) increased expression levels of NF-κB (Hajra *et al*, 2000); and iv) increased expression of VCAM-1 (Brooks *et al*, 2002). These results suggest that the defence mechanisms of endothelial cells may not always be able to cope with the entire range of shear stress encountered *in vivo*.

The influence of the vessel wall on coagulation occurring under flow conditions in polyethylene capillary tubes has also been studied. The ECM of endothelial cells activated to express TF by exposure to TNF-α was able to support FXa generation in the presence of FVIIa. This was blockable by TFPI or inactivated FVII (FVIIai) confirming that activation of the extrinsic pathway was occurring (van't Veer *et al*, 1994; Valentin *et al*, 1995). The ECM of the activated endothelial cells increased FXa generation 20-fold in comparison to the activated cells, indicating that even activated endothelium is much less procoagulant than the matrix that is exposed following vessel wall damage (Lindhout *et al*, 1992). Similar results have been obtained with inactivated Factor IX (IXai), indicating that the intrinsic pathway also contributes to coagulation in the presence of activated endothelium and its ECM (Tijburg *et al*, 1991). Experiments investigating the effect of varied shear rates on coagulation during the perfusion of blood over collagen, a component of ECM, or activated endothelial cells found that fibrin thrombi formed on procoagulant endothelial cells with most deposition occurring at low shear rates, whereas the collagen surface encouraged platelet deposition at high shear rates (Diquelou *et al*, 1995a). These studies confirm the important influence of shear stress on the condition of the endothelium or its matrix, and the important influence that this has in turn on the coagulation process.

1.3 Summary and aims of thesis

Active processes that involve numerous components of plasma, platelets and endothelial cells combine to keep the blood fluid and prevent unwanted coagulation that may occlude vessels and impair the circulation. However, when the vessel wall is damaged, the highly complex coagulation system reacts rapidly to prevent blood loss, with explosive generation of thrombin leading to a platelet plug and then a fibrin clot. Built in to this system are inhibitory pathways that switch off the coagulant response in order to prevent unlimited growth of the clot. All these processes occur as the blood is flowing through the vessels that are in need of repair, both delivering and washing away the components of the response. Platelets are effectively captured on to damaged surfaces where they then provide a catalytic surface for the interaction of coagulation enzymes that eventually form the fibrin clot. Downstream of the site of damage, any excess thrombin that is washed out of the clot is captured and inhibited by thrombomodulin on endothelial cells, where it becomes able to inactivate coagulation cofactors, thus preventing extension of the clot.

Antithrombotic drugs are used to treat and prevent thrombosis, with the aim of preventing unwanted coagulation but without compromising haemostasis. Inhibitors of specific coagulation factors such as heparin, low molecular heparin and hirudin are widely used to dampen the coagulation response in at-risk individuals. Anti-platelet antibodies are a relatively new class of drug that are under investigation as inhibitors of adhesion and aggregation of platelets and for their apparent anticoagulant activity.

It is clearly important that these drugs are able to function under the precise conditions that they will encounter in the body, where endothelial cells, plasma and platelets are interacting under flow conditions. The aim of this thesis is to establish a test system that can mimic these conditions in the laboratory and to examine several classes of drug in order to elucidate their relative effectiveness under the flow conditions that may be found in different sized blood vessels, and in the presence of intact or damaged endothelium.

CHAPTER 2

MATERIALS & METHODS

2.1 Introduction

The design, procurement and assembly of the flow system followed by the assessment and validation of its performance are described in detail in Chapter 3. The results of the studies on antithrombotic agents are detailed in Chapters 4 and 5. Certain materials and methods are common to all three chapters and to avoid repetition their details are presented together in this chapter.

2.2 Buffers

Tris-buffered saline (TBS) was used as the dilution and washing buffer for all experiments. It comprised: 0.05 M Tris (hydroxymethyl) aminomethane, 0.15 M NaCl, pH 7.4 (BDH reagents from Merck, Poole, UK).

Thrombin generation was measured in samples taken into a collection buffer which comprised: 0.05 M Tris (hydroxymethyl) aminomethane, 0.175 M NaCl, 0.0075 M ethylenediaminetetraacetic acid disodium salt dihydrate ($Na_2EDTA.2H_2O$; all chemicals from BDH as before), pH 8.4 with 1% v/v human serum albumin (HSA; from BPL, Elstree, UK).

2.3 Tissue factor

RecombiPlasTin®, recombinant tissue factor (TF) from Ortho Diagnostics, Raritan, NJ, USA, reconstituted with water. Each batch was calibrated in a prothrombin time (PT) assay against an in-house standard (NIBSC preparation 94/738) that had been calibrated against American Diagnostica Lipidated Recombinant Tissue Factor (Product 4500L; Greenwich, CT, USA) as follows. Doubling dilutions of the

RecombiPlasTin® and American Diagnostica TF preparations were prepared in TBS and 100 µl of each dilution was added to 100 µl of pooled normal plasma in a test cuvette of an Amelung KC-4A coagulometer (Brownes, Reading, UK). Following a 1 minute incubation at 37 °C, 100 µl of 25 mM $CaCl_2$ was added and the clotting time was recorded. Parallel line analysis (using NIBSC in-house software) was performed on duplicate determinations on linear sections of the dose response curves, and an accurate potency relative to the in-house standard could therefore be assigned to each batch of RecombiPlasTin®.

Two concentrations of TF were used in subsequent experiments and are referred to as 'high' and 'low' TF. Based on the product information from American Diagnostica, and using the data obtained from the calibration prodecure, the final concentration in the 'high' and 'low' TF reaction mixtures used were 630 and 35 pM, respectively. When these concentrations of TF were used in a PT, clotting times were 245 ± 4 and 339 ± 21, for 'high' and 'low' TF respectively (mean ± sd, n = 5).

2.4 Collection of blood

Blood was collected from healthy volunteers, who denied taking non-steroidal anti-inflammatory drugs in the previous seven days, by venepuncture of the antecubital vein. Blood was anticoagulated in the ratio of 8.5 ml of blood to 1.5 ml of acid-citrate-glucose formula A (ACD-A; 0.07 M sodium citrate, 0.04 M citric acid, 0.12 M glucose, pH 5.0).

2.5 Preparation of normal plasma pools

Blood was collected as above from a minimum of 20 randomly selected donors, who denied taking non-steroidal anti-inflammatory drugs in the previous seven days, and centrifuged at 30 000 g for 15 minutes at 20 °C within 20 minutes of collection. The plasma was carefully aspirated from between the cell layer and any floating lipid. The plasma was pooled and centrifuged at 17 000 g for a further 15 minutes at 4 °C and aliquots were snap frozen in liquid nitrogen and stored at –40 °C.

2.6 Defibrination of normal plasma

Defibrinated normal plasma (DNP) was prepared immediately before use. Aliquots of pooled normal plasma were thawed at 37 °C and defibrinated using ancrod (1st International Reference Preparation, 74/581) 0.5 IU per ml of plasma for 20 minutes at 37°C. The fibrin was removed by winding onto wooden sticks followed by centrifugation at 1 000 g for 5 minutes. Other methods of defibrination were also investigated and are discussed in Sections 4.2.6 and 4.3.8.

2.7 Preparation of 'platelet plasma'

Group O donors were selected for platelet donation in order to avoid reaction of anti-A and anti-B antibodies in the pooled normal plasma with the donor platelets. Blood was collected as above and centrifuged at 150 g for 10 minutes. The resulting layer of platelet rich plasma (PRP) was carefully aspirated and acidified by the addition of 1/10 volume of ACD-A. The PRP was then centrifuged at 1 000 g for 5 minutes to sediment the platelets. The platelet poor plasma was removed and the platelets were resuspended in 1 ml of DNP. The platelet count was measured using a

Coulter Act-T 8 cell counter (Beckman-Coulter, High Wycombe, UK) and adjusted by the addition of more DNP to give the required count. This is referred to as 'platelet plasma' (PP). A previous study (Reverter *et al*, 1996) found 300 x 10^6 platelets/ml to give near maximal thrombin generation in a static system and this count was therefore adopted in these studies, although different results were found in the flow system (see Section 3.3.3.2). The platelet count was adjusted to 375 x 10^6 platelets/ml, giving a final concentration in the reaction mixture of 300 x 10^6 platelets/ml. The normal range in blood is 150 – 400 x 10^6 platelets/ml (Kay, 1998).

2.8 Thrombin generation test

In order to measure the effects of the antithrombotic agents to be studied, a suitable assay was needed. *In vitro* diagnostic tests that are used in the clinical laboratory are usually based on the clotting time (or the chromogenic activity equivalent) of a sample of citrated platelet poor plasma. These tests, such as the prothrombin time (PT) and the activated partial thromboplastin time (APTT) are useful as indicators of specific deficiencies or inhibition. The PT detects deficiencies in the extrinsic or common pathway by activating coagulation with tissue factor and calcium ions, and prolongation of the clotting time indicates a deficiency of any of fibrinogen, prothrombin, or FV, FVII, FIX or FX. The PT may also detect the use of oral anticoagulants such as warfarin, which results in incomplete synthesis of the vitamin K dependent clotting factors mentioned. In the APTT, the intrinsic pathway of coagulation is initiated by a 'surface activator', such as kaolin, in the presence of phospholipid and calcium ions. A prolonged clotting time may indicate a deficiency in any clotting factor except FVII and the APTT is frequently used to monitor

heparin therapy, although its relevance for this has been questioned (Kay, 1998; Hirsh *et al*, 2001). The endpoint of both of these tests is the formation of a fibrin clot, which is the result of the generated thrombin cleaving fibrinogen. The concentration of thrombin required for this is around 20 nM or 2 international units (IU) per ml and this concentration is achieved very quickly following activation of coagulation.

The PT and APTT have been referred to as measures of the lag time of thrombin generation, where trace amounts of thrombin activate FV, FVIII and FXI in a positive feedback loop that amplifies the coagulation response (Hemker, 1994). However, it may be the case that sufficient thrombin is generated to produce a clot without positive feedback and that these tests measure only the first phase of prothrombin conversion to thrombin and not the second, amplified phase. The insensitivity of the PT to heparin is an indication of this, as inhibition of the feedback activation of FV by thrombin would lengthen the clotting time if the second phase of thrombin generation were occurring.

The coagulation process does not end with the production of a fibrin clot, and thrombin continues to be formed, to concentrations as high as 250 nM or 25 IU/ml, driving the second phase of the coagulation response with positive feedback effects. In addition to its procoagulant effects thrombin also activates Protein C and, indirectly, the TFPI inhibitory pathway. These inhibitory mechanisms switch off the coagulation process and allow the circulating inhibitors of thrombin (antithrombin, $\alpha_2$macroglobulin and α_1-antitrypsin) to neutralize the remaining thrombin and bring the process of coagulation to an end (Hemker, 1994). The amount of thrombin that

can be detected at any point during the coagulation process therefore represents the balance between thrombin that is being generated and that which has been inhibited.

The curve that can be plotted of the concentration of thrombin against time provides information on several parameters of the coagulation process (see Figure 2.1). The area under the curve (AUC) indicates the total amount of thrombin that has been able to contribute to the promotion and inhibition of coagulation. The AUC is expressed in IU.seconds/ml, taking into account both the concentration of thrombin and the time for which it is active – this can be compared to the 'man-hours' of work that the enzyme is able to perform. The thrombin generation curve may be affected in different ways by the presence of different inhibitors such as heparin and hirudin. The height of the curve at its peak and the time at which it occurs also provide information regarding the progression of the first and second phases of the coagulation response and its natural or artificial inhibition (Hemker, 1994).

The thrombin generation test was therefore considered to be the most appropriate test to use for the study of coagulation and its inhibition under flow conditions, due to its provision of data covering the whole coagulation process rather than the single 'snap-shot' view provided by clotting tests such as the PT and APTT. The flow experiments were designed to allow the collection of timed subsamples for thrombin determination at the outlet of the flow chamber, and this collection regime was reproduced with manual subsampling in static experiments.

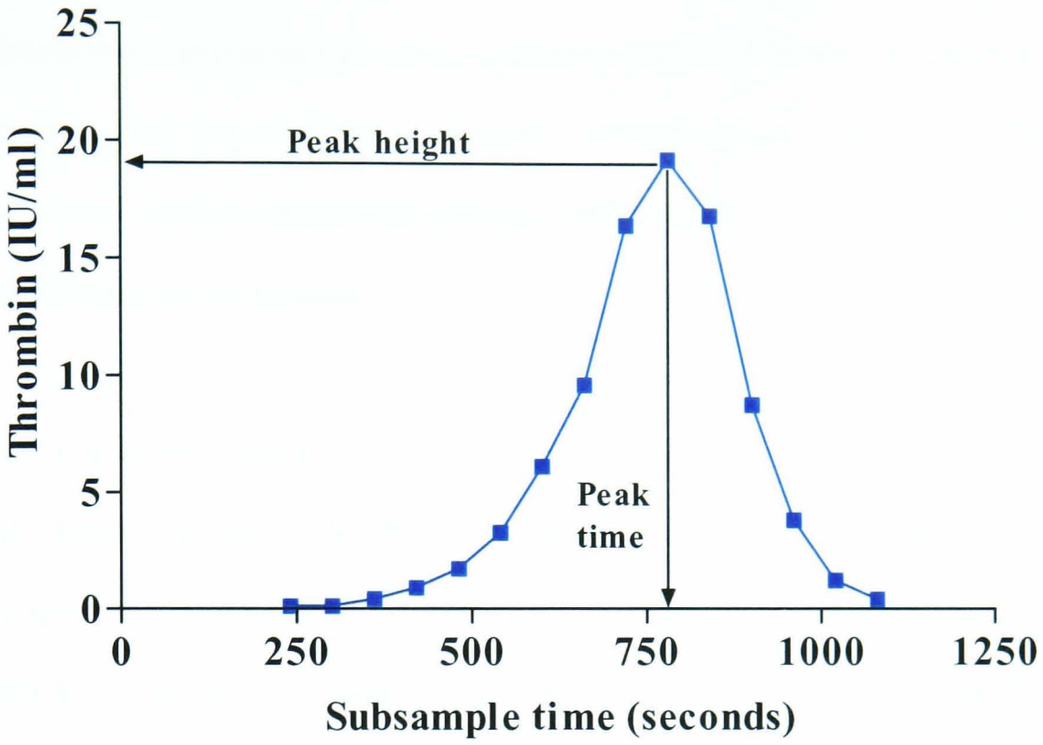


Figure 2.1 *Thrombin generation curve.*

The curve shows data from a representative static experiment performed using the clotting method. The total amount of thrombin generated can be calculated from the area under the curve. The height of the curve at its peak and the time at which this occurs are also useful indicators of the coagulation process.

2.8.1 Reaction mixture

2.8.1.1 Intrinsic method

This method was only used in the study of anti-platelet antibodies (see Chapter 5). 800 µl of platelet plasma was placed in a polystyrene tube (Depex brand, Deltalabs, Barcelona, Spain) to which was added 25 µl of TBS, or TBS containing the antibody to be studied, and 100 µl of 100 mg/ml kaolin (Sigma, Poole, UK) suspension in TBS. This mixture was incubated at 37°C for 20 minutes in order to allow antibody binding to platelets to reach equilibrium, and for contact activation of coagulation to occur. Thrombin generation was initiated by the addition of 75 µl of 200 mM $CaCl_2$ to give a final concentration of 15 mM. This concentration of calcium is equal to the

amount of calcium chelated by the citrate anticoagulant, resulting in an approximately physiological system and is similar to that used in previous studies (Reverter *et al*, 1996; Herault *et al*, 1998). In some assays, the volume of reagents used varied, but the final concentrations were unaffected and plasma was always 80 % of the reaction mixture.

2.8.1.2 Extrinsic method

This method was used in the study of anti-platelet antibodies (see Chapter 4), heparin and hirudin (see Chapter 5). The same principle was followed as the intrinsic method, but the platelet plasma was incubated with the inhibitor alone for 30 minutes (during the subsampling period of the previous assay). Although unlikely to be necessary for the binding of heparin or hirudin to platelets, in order to maintain consistency with the studies of anti-platelet antibodies, all experiments retained this pre-incubation. Thrombin generation was initiated with 175 µl of recombinant tissue factor diluted in 100 mM $CaCl_2$ (TF/Ca^{2+}). Tissue factor was used at final concentrations of either 630 or 35 pM ('high' or 'low', as described in Section 2.3) and $CaCl_2$ was at a final concentration of 17.5 mM. Assays were also performed in the absence of platelets using the lower concentration of TF.

2.8.2 Static conditions

In experiments that included HUVEC or ECM, following the addition of TF/Ca^{2+} to the plasma/buffer/inhibitor mixture, the reaction mixture was added to a culture well containing the washed HUVEC or ECM. The culture plate and the 96 well sample plate (Greiner, Frickenhausen, Germany) were maintained at 37 °C using heating blocks (Techne, Cambridge, UK). In experiments that did not include HUVEC or

ECM, the reaction mixture remained in the polystyrene tube. In some experiments with high concentrations of inhibitors, the start of subsampling was delayed by up to 30 minutes, as no thrombin generation was seen during this period in preliminary experiments. The time course was up to 64 minutes, with subsamples being taken at 60 second intervals. Thrombin was measured as described below.

2.8.3 Flow conditions

Thrombin generation was initiated by the addition of TF/Ca^{2+} to the plasma/buffer/inhibitor mixture, and the reaction mixture was then aspirated into a syringe, connected to the flow chamber and mounted on the syringe pump. The syringe pump and flow chamber were mounted on an XYZ-translation table that was set to collect subsamples at 60 second intervals for between 32 and 64 minutes. The entire system was housed in a custom-built incubator set to 37°C. For full details of the flow system, see Section 3.2.

2.8.4 Thrombin detection

Two methods were used to detect the concentration of thrombin in activated plasma, based on clotting or chromogenic assay systems (summarised in Figure 2.2). The clotting method was used in the first set of static experiments that studied anti-platelet antibodies. These experiments were performed before the software required for interpretation of the chromogenic data had been written. Subsequent experiments, and all of the flow experiments, used the chromogenic system.

2.8.4.1 Clotting system

Fifty µl subsamples of the activated plasma were taken every 60 seconds into 200 µl of fibrinogen solution (Diagen bovine fibrinogen, Diagnostic Reagents, Thame, UK) and the clotting time measured using Amelung KC-4A coagulometers (Brownes, Reading, UK). The clotting times were compared with a standard curve prepared with the fibrinogen solution and the 1st International Standard for human α thrombin (89/558, NIBSC, UK) in order to determine the concentration of thrombin in each subsample.

2.8.4.2 Chromogenic system

Subsamples of either 20 or 30 µl were taken into 150 µl of EDTA-stop buffer. This buffer chelates the calcium in the reaction mixture, preventing any further thrombin generation from occurring. At the end of each time course the thrombin concentration of the subsamples in collection buffer was assayed by the addition of 40 µl of 3 mM chromogenic substrate S2238 (Chromogenix-Instrumentation Laboratory, Milan, Italy) at 37°C to each well. Colour development was allowed to proceed for 3 minutes before the reaction was stopped using 80 µl of 50 % (v/v) acetic acid (Merck, Poole, UK) and the optical density read at 405 nm using a Thermomax platereader (Molecular Devices, Menlo Park, CA, USA).

The optical density data were converted into thrombin units using standard curves prepared with the 1st International Standard for human α-thrombin (89/588, NIBSC, UK). As the biologically inactive thrombin-$\alpha_2$macroglobulin complex can still cleave this substrate, the data were then analysed using a program that calculates and subtracts the amidolytic activity of this complex (Hemker *et al*, 1986). Details of the

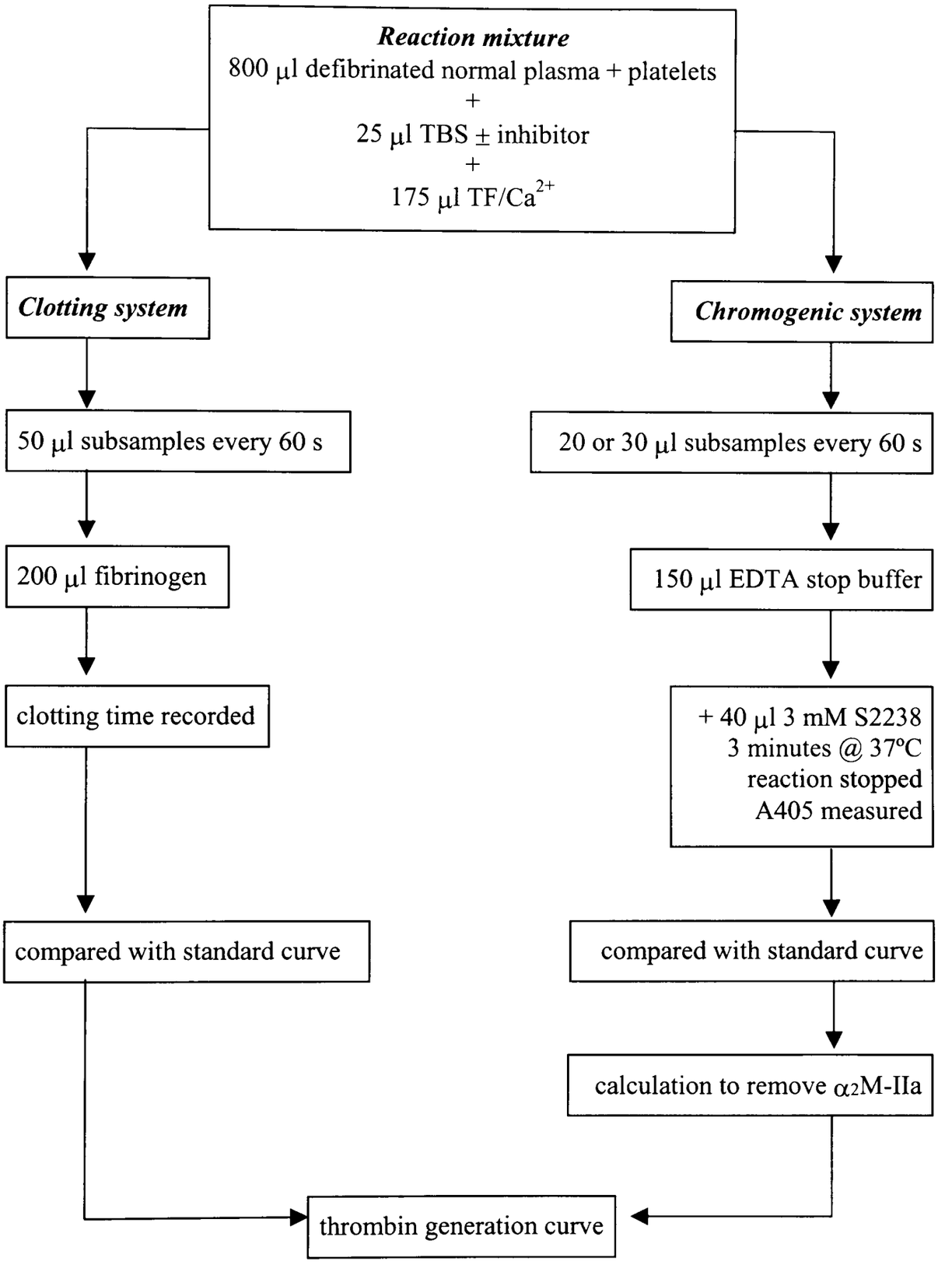


Figure 2.2 *Flow chart to illustrate clotting and chromogenic thrombin generation test methods*

calculations for the chromogenic correction and the area under the curve are found in Appendix 1, and an example of the corrected chromogenic thrombin generation curve is shown in Figure 2.4.

Despite the fivefold dilution and the change in pH to 8.4, there is a possibility that further inhibition of free thrombin may continue in the stop buffer prior to chromogenic thrombin determination. Assays were therefore performed where subsamples were taken both into stop buffer (for chromogenic thrombin determination at the end of the experiment) and into buffer that contained the chromogenic substrate (with the reaction being allowed to proceed for 3 minutes before stopping with acetic acid as before). Thrombin generation was initiated with low TF in a reaction mixture that contained 0, 0.125 or 0.25 IU/ml UFH, the highest concentration being sufficient to inhibit AUC by more than 50 % in the presence of HUVEC under static conditions (see Table 5.1, Chapter 5). Heparin would continue to potentiateany inhibition by antithrombin that was occurring in the stop buffer, and the lag time (defined as the time to reach 0.5 IU/ml thrombin) would therefore be longer in the stopped samples than in those assayed immediately. No significant differences in the lag time were seen between the stopped and immediate assays (Figure 2.3). In addition, there were no differences in the area under the curve, the peak thrombin concentration or the time of the peak. This indicates that there was no further inhibition of thrombin by antithrombin in the stopped chromogenic system.

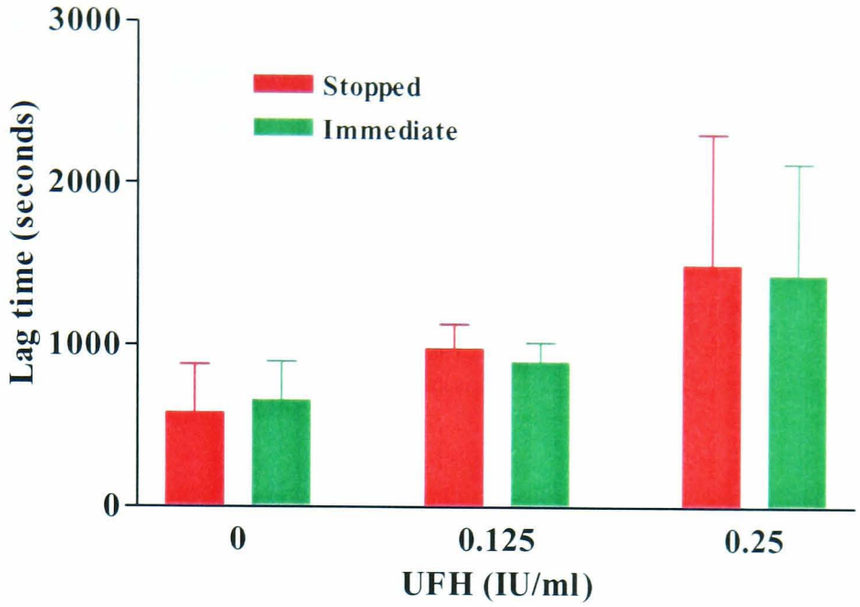


Figure 2.3 *Stopped or immediate chromogenic determination of thrombin concentration.*

Defibrinated plasma containing 300 x 10^6 platelets/ml was preincubated with varied concentrations of unfractionated heparin and thrombin generation was initiated with 35 pM TF and calcium. The time at which 0.5 IU/ml thrombin was first detected was defined as the 'lag time'. Mean ± standard deviation, n = 3, no significant differences.

2.8.5 The thrombin generation curve

Thrombin generation curves were prepared by plotting the thrombin concentration of subsamples against the time at which they were taken. The area under the thrombin generation curve (AUC) was calculated as a measure of the total thrombin generation, and has the units "IU.seconds/ml". The height of the thrombin generation curve at its peak and the time at which the peak occurred were also recorded. The amount of thrombin generated by platelet plasma with TBS and no inhibitors was taken to be 100 % in each case. An example of a thrombin generation curve from a clotting assay is shown in Figure 2.1 and from a chromogenic assay in Figure 2.4.

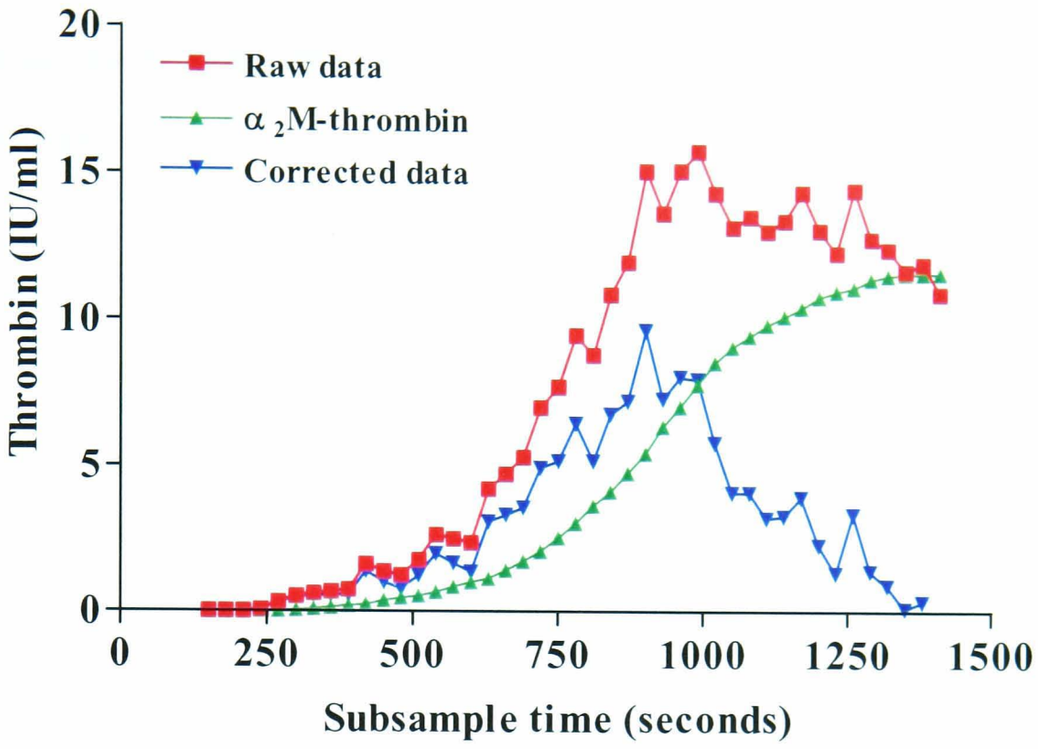


Figure 2.4 *Thrombin generation curve from a chromogenic assay.*

The raw data are analysed and the component that is due to the activity of the biologically inactive $\alpha_2$macroglobulin-thrombin complex is calculated and subtracted to give the corrected data.

2.8.5.1 Comparison of clotting and chromogenic methods of thrombin detection.

The clotting and chromogenic methods for thrombin detection were directly compared in a series of experiments. Subsamples were taken from a single reaction mixture, following low TF stimulation, into either fibrinogen or stop buffer for chromogenic determination.

The thrombin generation curves plotted from clotting and chromogenic determinations were different in shape, with a significantly higher thrombin peak seen in the clotting system than the chromogenic system (22.0 ± 2.6 and 8.2 ± 0.5 IU/ml respectively, mean ± standard deviation, $n = 3$; $p < 0.05$; Figure 2.5). The difference in the area under the curves was not significant (7611 ± 1497 and

4581 ± 681 IU.seconds/ml for clotting and chromogenic respectively), and neither was the time of the thrombin peak (630 ± 108 and 740 ± 195 seconds for clotting and chromogenic respectively). The lower peak of thrombin generation curves prepared from a chromogenic system compared to a clotting system has been noted previously. The presence of EDTA was identified as the cause of the delay, and the inhibitory effect of EDTA on the thrombin – S2238 reaction suggested as a reason for the lower peak, its effect being amplified by the high dilution of the subsample in the buffer (Houbouyan *et al*, 1996).

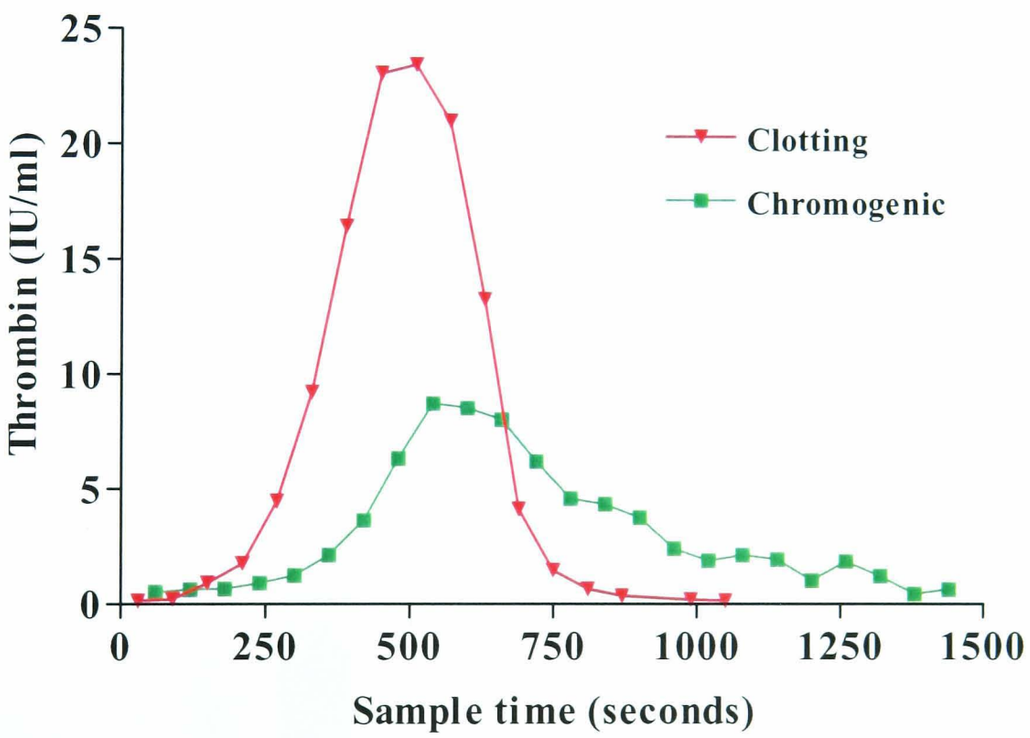


Figure 2.5 *Thrombin generation curves to compare clotting and chromogenic methods of thrombin detection.*

Data are from a single experiment where thrombin generation was initiated in defibrinated plasma containing 300 x 10^6 platelet/ml with 35 pM TF and calcium. Timed subsamples were taken from the plasma into both fibrinogen and stop buffer, for clotting and chromogenic determination of thrombin concentration, respectively.

When the thrombin generation test is used to analyse the effect of inhibitors, each experiment includes a curve prepared in the absence of any inhibitor. The measurements of this curve are defined as 100 % and the curves prepared in the presence of inhibitors are measured against it. This means that data may be compared across experiments and that variations in the profile of the curves in each experiment are controlled for. In order to confirm this, concurrent clotting and chromogenic determinations were made on subsamples from a reaction mixture containing 20 μg/ml of RFGP56, an inhibitory antibody (see Chapter 4). No significant differences were seen in the AUC, peak thrombin concentration and time of peak thrombin between the clotting and chromogenic systems (Figure 2.6).

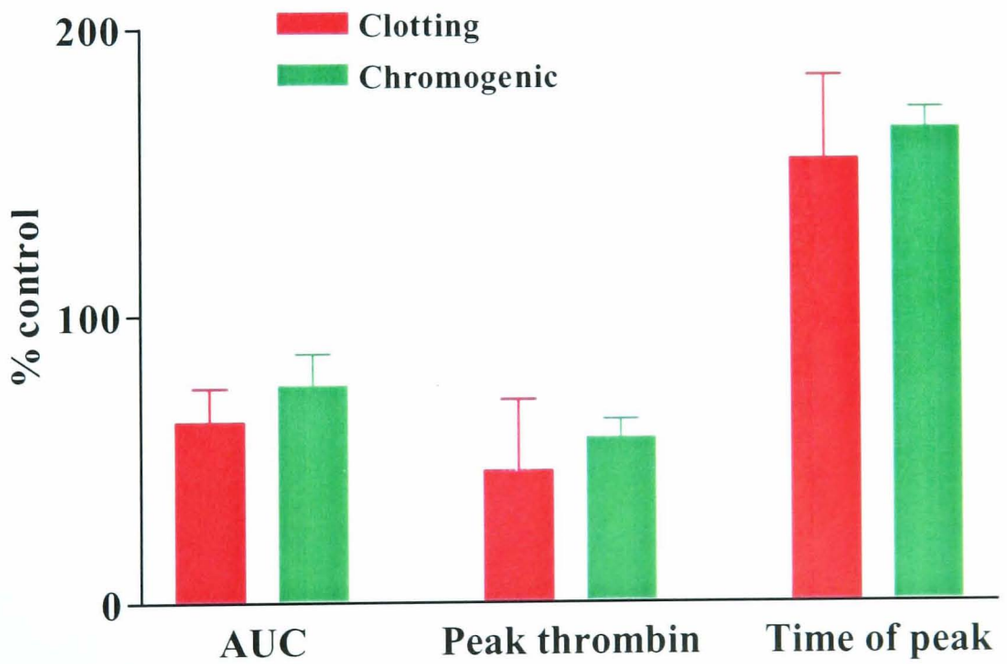


Figure 2.6 *Inhibition of thrombin generation measured by clotting and chromogenic methods. Defibrinated plasma containing 300 x 10^6 platelets/ml was preincubated with 20 μg/ml RFGP56 and thrombin generation was initiated with 35 pM TF and calcium. Timed subsamples were taken from the plasma into both fibrinogen and stop buffer, for clotting and chromogenic determination of thrombin concentration, respectively. Mean ± standard deviation, n = 3, no significant differences.*

2.9 Endothelial cell culture

Fresh human umbilical cords were collected (with ethical committee approval and patient consent) from Queen Elizabeth II Hospital, Welwyn Garden City, UK and from Chase Farm Hospital, Enfield, UK. Human umbilical vein endothelial cells (HUVEC) were harvested using a 1 % (w/v) collagenase A (Roche Diagnostics GmbH, Mannheim, Germany) solution in Medium 199 (Sigma, Poole, UK) as previously described (Jaffe *et al*, 1973). The cells were cultured in RPMI 1640 growth medium (Gibco, Life Technologies, Paisley, UK) supplemented with 15% heat-inactivated human serum (group AB, pooled from at least 4 donations, Welsh Blood Service, Cardiff, UK). First passage cells were used, and were cultured in Falcon 24 well plates (Meylan, France) for static experiments or on 10.5 x 22 mm Thermanox coverslips in 8 well plates (both Nalge Nunc International, from Life Technologies, as before). The cells were used when they had become confluent, usually at 3-5 days after seeding. The culture medium was aspirated and the cells gently washed with warm TBS before use.

2.10 Preparation of ECM

Two methods for the preparation of ECM were investigated. In both cases, HUVEC were allowed to grow for 5 - 7 days after seeding. The culture medium was aspirated and the cells were removed either by blotting them with a polyvinylidene (PVD) membrane (Immobilon-P, Millipore, Watford, UK) as described by Aznar-Salatti *et al* (1991) or using a cell scraper (Falcon, Meylan, France). The ECM was then washed with warm TBS and the removal of endothelial cells confirmed by light microscopy. The quality of the ECM was investigated by immunofluorescent staining with rabbit polyclonal antibodies to human vWF or fibronectin and a fluorescein isothiocyanate conjugated (FITC) swine anti-rabbit IgG (all antibodies from Dako, Glostrup, Denmark).

It was noted that a more even covering of vWF and fibronectin was present on coverslips prepared using the membrane method (as shown in Figure 2.7) than the cell scraper (not shown). The membrane method of cell removal was therefore chosen for use in future experiments.

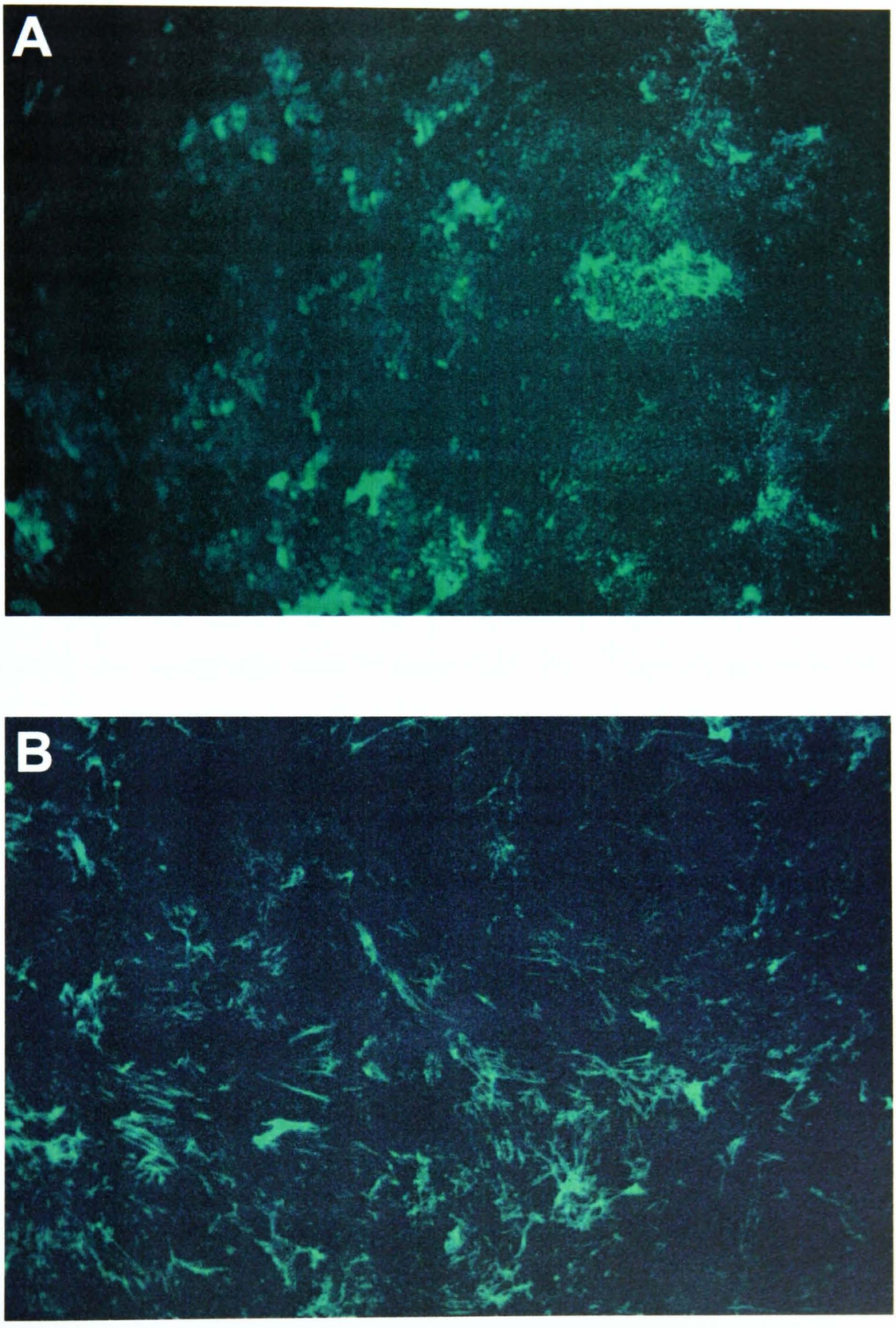


Figure 2.7 *Immunofluorescent staining of extracellular matrix.*

ECM was prepared by blotting with PVD membrane. Polyclonal rabbit antibodies to A) vWF or B) fibronectin were added and detected with FITC swine anti-rabbit IgG polyclonal antibodies, as previously described (Aznar-Salatti et al, 1991).

2.11 Immunofluorescent staining of platelets and HUVEC

In order to visualise the effects of shear stress on endothelial cells and platelets (Chapter 3) and the binding of antibodies to platelets and HUVEC (Chapter 4), immunofluoresence was performed as follows.

For studies of platelets in suspension, platelet plasma was prepared as detailed in Section 2.7 and incubated for 30 minutes at 37 °C with 20 μg/ml of the primary antibody, diluted in 6-salt PBS (137 mM NaCl, 3 mM KCl, 9 mM $CaCl_2$, 5 mM $MgCl_2$, 80 mM Na_2HPO_4 and 10 mM K_2PO_4; all reagents from BDH, Poole, UK) . A drop of the cell suspension was then applied to a glass coverslip that had been pre-treated with 10 mg/ml poly DL lysine (Sigma, Poole, UK). After 30 minutes, the coverslip was washed with 6-salt PBS and fixed with 2 % paraformaldehyde (BDH, Poole, UK) in 6-salt PBS for 15 minutes at room temperature. After fixing, the coverslips were washed in 6-salt PBS including 0.1 % glycine (to quench aldehyde groups; BDH, Poole, UK). HUVEC were cultured on Thermanox coverslips as described in Section 2.9, washed, fixed and incubated with antibodies, as before. The coverslips were then incubated for 60 minutes at room temperature with a 10 μg/ml solution of FITC secondary antibody in 6-salt PBS + 1 % v/v Triton X-100 (Sigma, Poole, UK), and 66 nM Alexa Fluor 532 phalloidin (Molecular Probes, Eugene, USA) to stain actin. Coverslips were washed three times in 6-salt PBS, once in 6-salt PBS containing the nuclear stain Hoechst 33342 (Molecular Probes, as before) and washed three further times in 6-salt PBS.

The fixed and stained cell preparations were examined and images captured using a Leica TCS SP2 confocal microscope with an acoustic optical beam splitter (Leica

Microsystems AG, Wetzlar, Germany), operated by Dr Roland Fleck of the Cell Biology and Imaging Section, NIBSC.

2.12 Statistical analysis

All data quoted are from $n \geq 3$ and are arithmetic mean ± standard deviation, unless otherwise stated. Statistical significance was determined using one-way analysis of variance (ANOVA) with Dunnett's post test using GraphPad InStat version 3.05 for Windows 95, (GraphPad Software, San Diego, USA).

Mr Peter Rigsby of the Informatics Laboratory, NIBSC performed all comparisons of AUC, peak-height and time of peak values by ANOVA and the calculations of IC_{50} data, which were calculated from weighted regression of logit response on log dose.

CHAPTER 3

THE FLOW SYSTEM

3.1 Introduction

The majority of *in vitro* studies of blood coagulation have been performed under static conditions in test tubes where the fluid dynamics and the flux of substrates and products that result from *in vivo* blood flow are not present. These studies provide only limited information and different *in vitro* approaches are required to model the *in vivo* process of coagulation. The use of flow chambers has become widespread since the mid 1970s and a number of different approaches have yielded interesting data, as summarised in Section 1.4. It is now accepted that the flow of blood has effects on the cells of the vessel wall, on blood cells such as platelets, and on the rate at which fluid phase enzymes, substrates and products are delivered to or removed from sites of coagulation at the vessel wall.

This chapter will outline the physical effects of blood flow, the types of flow chambers that have previously been developed to study these effects, and the design, construction and validation of the NIBSC flow system.

3.1.1 Shear stress and shear rate

When a fluid of a suitable viscosity is pumped through a tube, laminar flow is observed. In laminar flow the fluid is considered to be behaving as a number of concentric cylinders slipping over each other (see Figure 3.1).

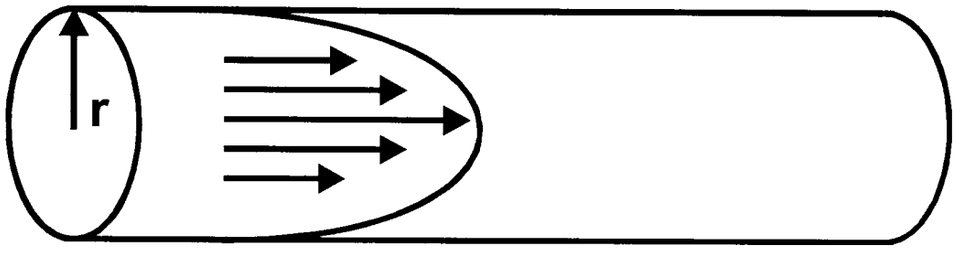


Figure 3.1 *Schematic of fluid flow through a cylinder.*
The velocity profile is parabolic and is zero at the vessel wall and highest at the centre line. r = radius of the cylinder.

The rate at which the layers of fluid slip over each other is known as the shear rate (γ). This is calculated as the derivative of the velocity profile dV/dr (where V is the flow velocity and r is the radius of the vessel lumen) and is expressed in inverse seconds (s^{-1}). The velocity profile of the fluid is such that the fluid in contact with the wall (the 'boundary layer') is considered to be stationary, whilst the fluid in the centre of the vessel flows the fastest. This means that the shear rate is greatest at the vessel wall, and zero at the centre of the vessel (Sakariassen *et al*, 2001). The shear rate at the vessel wall may be calculated using Equation 3.1.

$$\gamma = \frac{32Q}{\pi d^3}$$

γ = shear rate (seconds^{-1})
Q = flow rate (ml/second)
d = diameter of vessel (cm)

Equation 3.1 To calculate shear rate in a cylindrical vessel.

Another commonly used term is shear stress (τ), which is a measure of the force exerted on the adjacent lamina of fluid (or, in the case of the boundary layer, the force exerted on the vessel wall) by the flowing fluid. This is expressed as force per unit area, usually in dynes/cm^2 (where 1 dyne = 1 g.cm^{-1}s^{-2}). The viscosity of the fluid influences the shear stress that it exerts, and assumptions are often made regarding the viscosity of blood, which varies with haematocrit and temperature. Assuming physiological conditions of a 45 % haematocrit at 37 °C, a wall shear rate of 1500 s^{-1} would exert shear stress on the vessel wall of 57 dyn/cm^2 (Alevriadou & McIntire, 1995).

As described in Section 1.4.1, blood flow influences the interactions of clotting factors and inhibitors. The variety of flow rates, vessel diameters, occlusions and valves that are found throughout the vasculature mean that a wide range of shear rates and shear stresses are found at the vessel wall (see Table 3.1) and these have significant effects on the kinetics of blood coagulation.

Table 3.1 *Typical ranges of wall shear rates and shear stresses.*
(Goldsmith & Turitto, 1986; Alevriadou & McIntire, 1995)

Blood vessel	Wall shear rate (s^{-1})	Wall shear stress (dyn/cm^2)
Large arteries	300 – 800	11.4 – 30.4
Arterioles	500 – 1600	19.0 – 60.8
Veins	20 – 200	0.76 – 7.6
Stenotic vessels	800 – 10000	30.4 – 380

The variability of these conditions means that it is very difficult to mimic *in vivo* blood flow in an experimental environment but a number of devices have been developed that provide defined flow conditions. These devices may be used for *in vitro* studies, where an anticoagulant is usually present, or *ex vivo* studies using native blood, to investigate the influence of blood flow on coagulation.

3.1.2 Types of flow chamber

A number of different systems can be used to investigate the effect of shear on blood coagulation. Each has its advantages and disadvantages and a particular suitability to certain investigations depending on, for example, the need for continuous or endpoint sampling, the surface to be studied, the volume of perfusate available, *ex vivo* or *in vitro*.

Viscometers are probably the simplest system used for the study of the effects of shear on fluid phase reactants. The basic principle is of a rotating cylinder or cone contained within a stationary cylinder or dish. The 'Couette' viscometer consists of

coaxial cylinders and mimics the theoretical notion of shear stress generated between two flat plates of infinite size, one of which is stationary and one of which is moving with constant linear velocity thus generating a steady state fluid velocity profile (Slack & Turitto, 1994). A more widely used viscometer is the 'cone and plate' device where a rotating cone with a very shallow angle is lowered into a stationary flat-bottomed dish. The higher rotational speed of the outer region of the cone compensates for the increased gap between the cone and the plate, resulting in a near-constant shear stress throughout. The cone and plate viscometer has been widely used in the study of shear induced platelet aggregation and has also been used with endothelial cells grown in the plate (Malek *et al*, 1993). This system is not suitable for the continuous measurement of coagulation parameters as it is not possible to take samples of the medium during the course of the experiment.

Annular chambers have been used as a method to study coagulation in the presence of *ex vivo* vessels. In annular systems, an everted segment of a blood vessel is mounted on a central rod and surrounded by the outer cylinder of the perfusion chamber (Baumgartner *et al*, 1976; Weiss *et al*, 1991). Blood, or other perfusates, may then be passed through the chamber where the shear rate at the surface of the vessel segment is dependent upon the flow rate and the distance between the chamber wall and the segment surface. Different types of surface may be studied in this system, although it is unlikely that the process of everting the vessel would leave the endothelial cells intact, and studies of differing forms of subendothelial matrix are more common.

Tubular chambers work on a similar principle, but consist of a cylindrical chamber with a cut-out window through which a patch of test material is presented to the flowing medium. The material acts as a gasket to seal the window and this system is therefore unlikely to be suitable for the study of endothelial cells, as they would need to be cultured on to a flexible material and remain firmly attached when the material was under tension (Badimon *et al*, 1987).

A column-based flow system has been used to study the release of nitric oxide by endothelial cells exposed to shear stress. The cells were grown on microcarrier beads and then packed into a column, with the assumption that at a constant fluid viscosity the shear stress is a function of the column flow rate (Buga *et al*, 1991). While this may be a reasonable assumption, the system would be laborious to prepare and it would be difficult to establish the condition of the cells during and after a perfusion experiment, as damage may occur during the packing and unpacking processes.

Flow systems that use capillary tubes to mimic the *in vivo* environment have not been widely used. One group has succeeded in seeding endothelial cells into a capillary and studying their influence on coagulation (Lindhout *et al*, 1992). The drawback to this is that it is very difficult to establish whether or not a continuous monolayer of endothelial cells has been laid down within the capillary, and examination of the cells during or after the experiment can be difficult. In preliminary assessments of the flow system, similar problems were encountered and are described in Section 3.2.4.1.

An alternative to coating a capillary tube with endothelial cells is the use of phospholipid bilayers into which proteins of interest, such as tissue factor, have been inserted (Contino *et al*, 1991). This enables the influence of shear stress to be studied in a purified coagulation system. The small internal diameter of the tube means that relatively high shear rates can be generated with small amounts of medium and also that longer-term perfusions can be performed without recirculation or the consumption of precious reagents. Small volume samples can be collected at the outlet by 'plucking' drops of medium from the end of the capillary by touching the drop onto the meniscus of an aliquot of a collection buffer. Sample sizes as small as 6 μl have been reported using this technique (Contino *et al*, 1991). Timed samples may then be assayed for various parameters to determine the time course of coagulation.

Parallel plate systems are perhaps the most commonly used in the study of coagulation. They consist of two plates held parallel to each other with a narrow gap between them through which fluid is driven. When fluid with a constant viscosity is driven between the plates a parabolic velocity profile is seen, indicating that laminar flow is occurring (illustrated in Figure 3.2). The wide range of shear stresses that are experienced throughout the fluid means that these chambers are unsuitable for studying the effects of shear on cells or proteins in suspension. Analogous to parallel plate flow chambers are capillary tube microslides with a rectangular cross-section, which have been used to study platelet and neutrophil adhesion to endothelial cells (Cooke *et al*, 1993; Kirton & Nash, 2000).

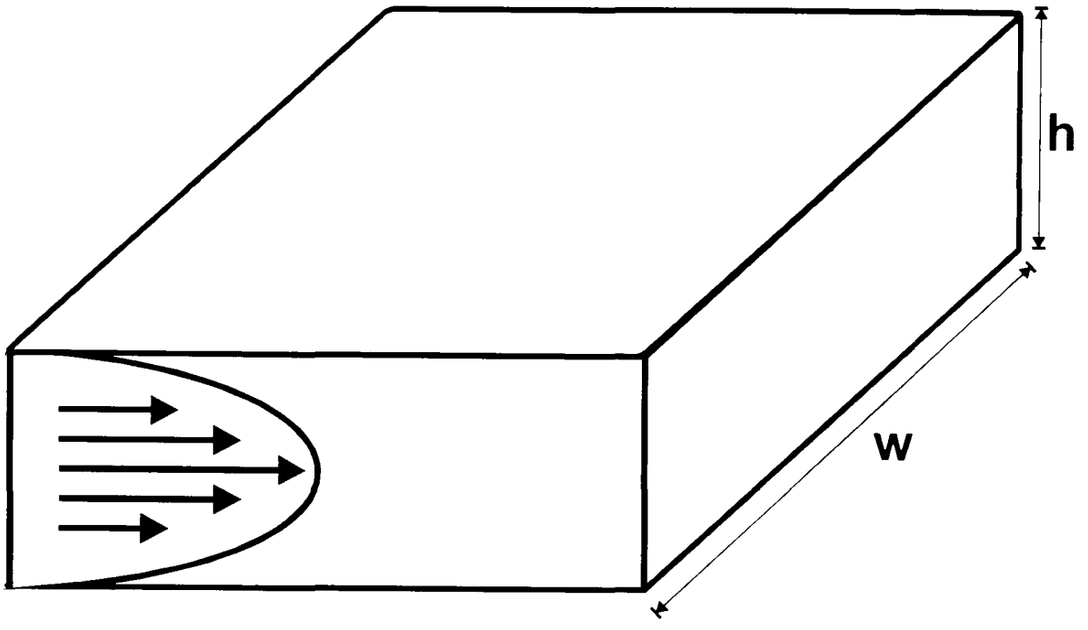


Figure 3.2 *Schematic of fluid flow through a parallel plate flow chamber.*
The plates are separated by distance h *and are* w *wide. The velocity profile of the fluid is parabolic.*

The height and width of the slit through which the fluid flows and the rate at which it is flowing determines the wall shear rate, which may be calculated using Equation 3.2. These chambers are therefore well suited to the study of shear on the delivery of suspended entities to the mass transfer boundary layer at the vessel wall. This is ideal for coagulation studies, where the influences of the endothelium and the subendothelium are important. The base of the chamber may be formed by a slide or coverslip onto which endothelial cells have been cultured or a specific matrix protein has been applied. The surface may be examined for morphological changes or adhesion of fluid borne elements after, and, depending of the construction of the chamber, during the perfusion. Sampling of the perfusate at the outlet of the chamber is straightforward, and small sample volumes may be collected using the 'plucking' method described above. This system does not mimic *in vivo* conditions

exactly, as the perfusate is only exposed to cells on one side of the chamber and an asymmetric flow velocity profile may be seen, but laminar flow is still considered to be present.

$$\gamma = \frac{3Q}{2wh^2}$$

γ = shear rate (seconds^{-1})
Q = flow (ml/second)
w = slit width (cm)
$h = \frac{\text{slit height (cm)}}{2}$

Equation 3.2 To calculate shear rate in a parallel plate flow chamber.

The parallel plate system has been further developed to include a stenosis that mimics those found in diseased vessels. These can be used to generate very high localised shear stresses and the stenosis can also be coated with material similar to that found in vessel wall plaques, allowing the effect of various antithrombotic drugs to be evaluated *in vitro* (Barstad *et al*, 1994). A chamber where the shear stress decreases linearly along the length of the chamber has also been developed (Usami *et al*, 1993). These adaptations are suitable for the study of adhesion of fluid phase reactants, such as platelets, but are not suitable for the study of the effects of specific shear rates on the perfusate.

The available volume of the perfusate has a significant bearing on the experiments that may be performed with particular flow chambers, as the flow rate of the fluid is critical to the shear rate generated. When fresh blood or its purified components are to be used, the volume that can be used in each experiment may be limited and this is particularly the case when a number of 'runs' are to be performed, for example when

preparing a dose-response curve (see Table 3.2 in Section 3.2.4.2). This problem may be overcome by recirculating the perfusate rather than pumping it straight through the chamber for collection of samples at the outlet. However, there are drawbacks to this method, as the concentration of soluble factors in the perfusate (released from activated endothelial cells or platelets) may rise above physiological levels and begin to interfere with normal reaction kinetics. The opposite effect may also occur, with depletion of soluble reactants, adhesion of platelets or product accumulation. Recirculating systems are useful in certain situations, such as in studies of gene regulation where long term exposure to flow is needed to allow transcription level effects to occur, but a single-pass system is preferable for the study of enzyme-based systems such as coagulation.

When a high shear rate is required, the alternative to a high flow rate is a smaller chamber. When parallel plate chambers were first developed, they had quite large internal dimensions, necessitating the use of large volumes of perfusate for realistic shear rates to be generated (Sakariassen *et al*, 1983). More recent studies have employed much smaller chambers that allow single-pass perfusions with the generation of realistic arterial shear rates (Usami *et al*, 1993; Sixma *et al*, 1998).

Taking into account all these issues, it was decided that the most suitable system for the analysis of thrombin generation under flow conditions was a parallel plate flow chamber with no recirculation of the perfusate. In order to generate a sufficiently high shear rate without using a large volume of plasma, flow chambers with small dimensions would be preferable. A suitable flow system was therefore designed and built for this project.

3.2 The NIBSC Flow System

The flow system was custom built using three major components (the syringe pump, the table and the incubator) and a number of other components that were used in the assembly of the system.

3.2.1 Syringe pump

The Harvard 33 double syringe pump (Harvard Apparatus, Edenbridge, Kent, UK), shown in Figure 3.3, is capable of pumping two syringes independently with flow rates from 0.0073 µl/hour to 53 ml/minute. It is pre-programmed to calculate flow rates from the diameter of the syringes used. An additional benefit of this pump is the ability for remote control using a personal computer and the RS-232C port, allowing it to be synchronised with the XYZ table.

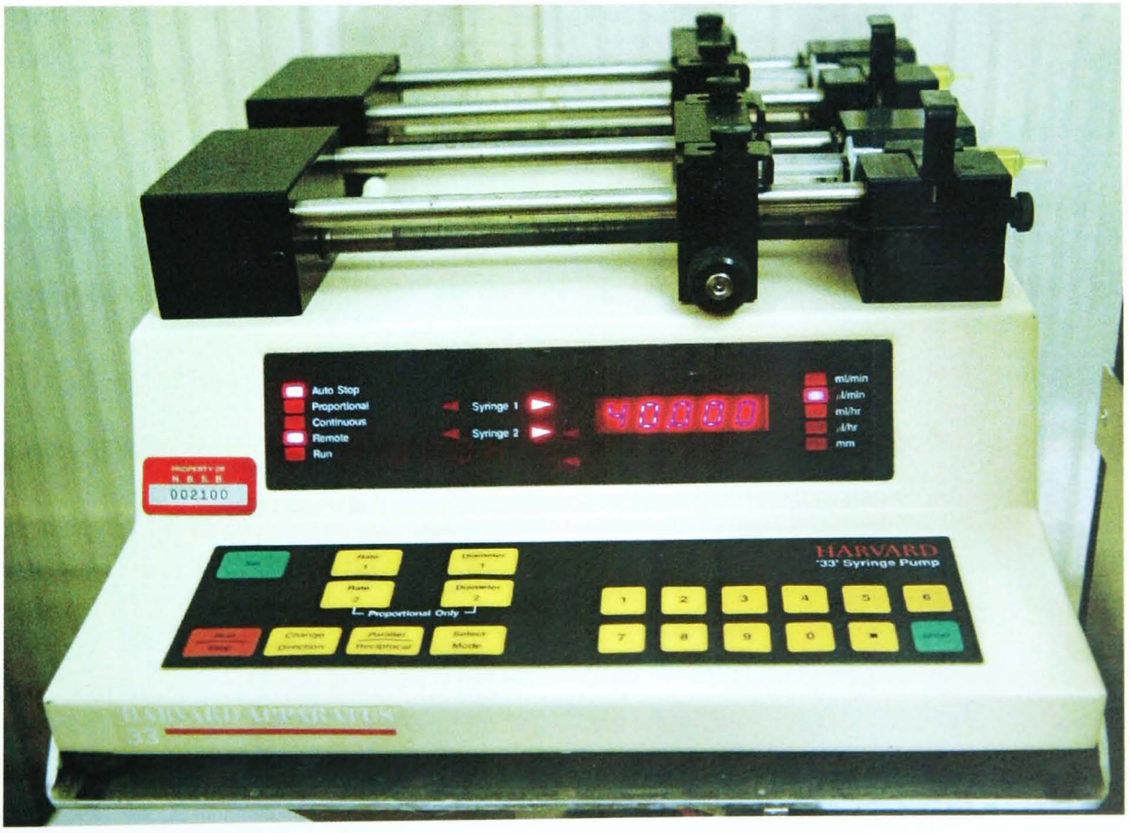


Figure 3.3 *The Harvard 33 double syringe pump.*

3.2.2 XYZ translation table

The Techno Isel 'Da Vinci' XYZ table (Unimatic Engineers, London, UK), shown in Figure 3.4, is a precision machine tool designed for use in industrial applications such as computer aided machining, engraving and other functions. For these applications, the appropriate tool is mounted to the Z-axis and the movement of this tool in three dimensions is controlled via the serial port of a personal computer. For construction of the flow system, the parallel plate flow chamber is mounted to the Z-axis in a vertical orientation. The 96 well plate into which samples of perfusate are to be collected is placed onto the X-axis. As the perfusion experiment progresses, precisely timed movements of both the X- and Y-axes position the outlet tubing of the chamber over subsequent wells of the collection plate. Movement of the Z-axis at the end of each sampling period enables the outlet tubing to be dipped into the meniscus of the collection buffer in the wells of the plate, stopping the reaction and allowing the collection of samples less than the size of one drop (see Figure 3.5). The reproducibility of the smallest sample volume used (20 μl) was determined by chromogenic analysis of 30 samples of a standard solution of 10 IU/ml thrombin (NIBSC reagent 94/708) and the coefficient of variation (CV) was found to be 4.3 %. The syringe pump was mounted on a shelf attached to the Z-axis, with tubing of sufficient length to connect the syringes to the inlets of the flow chamber.

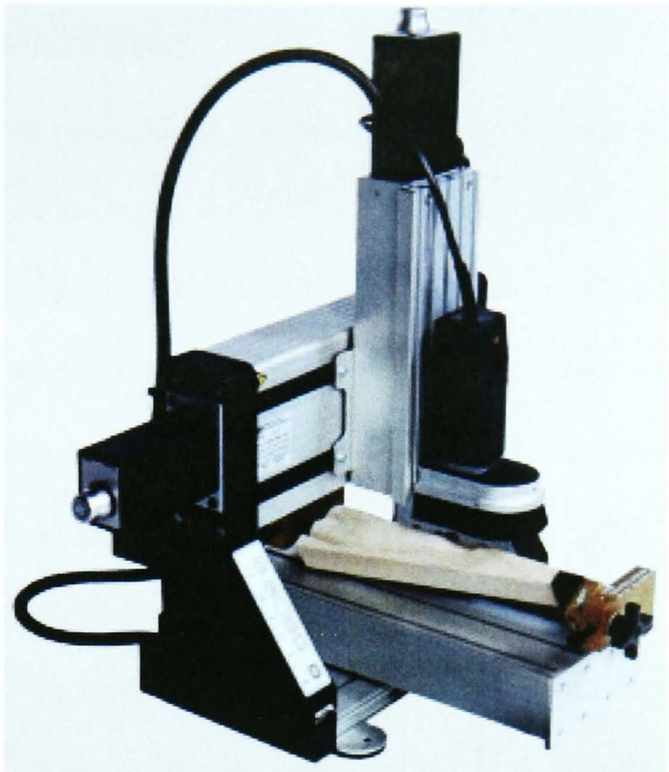


Figure 3.4 *The Techno Isel 'Da Vinci' XYZ table.*

Photograph provided by Techno Inc, New York, USA.

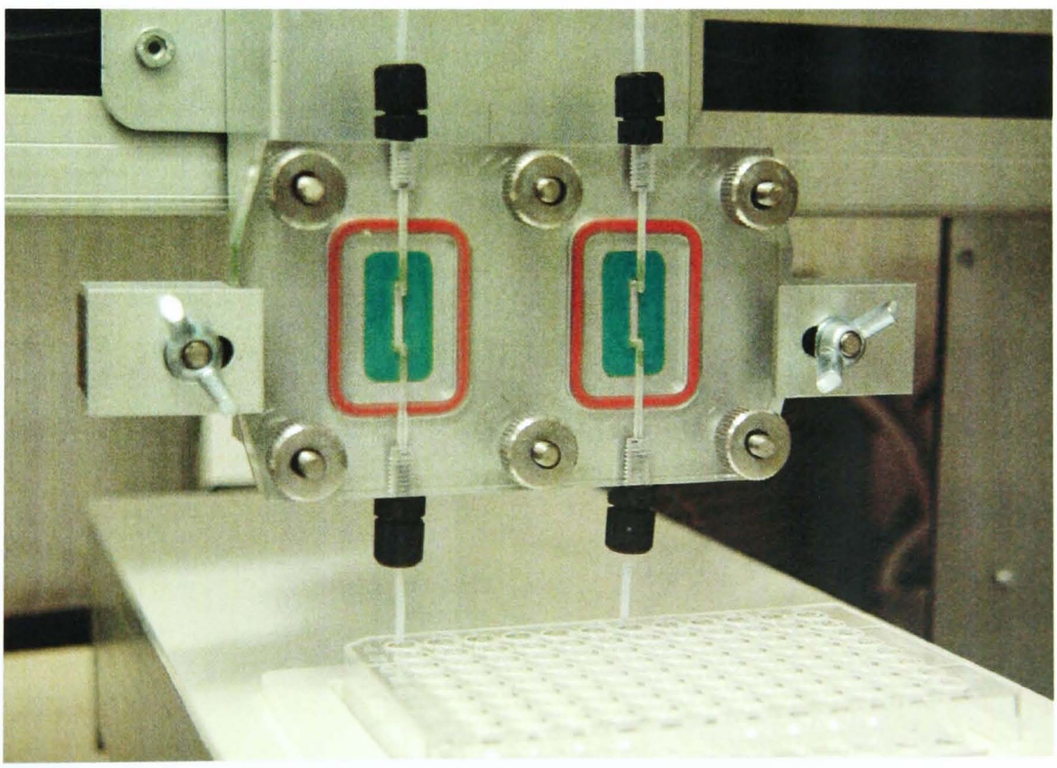


Figure 3.5 *The parallel plate flow chamber mounted to the Z-axis of the XYZ table.*

Timed subsamples are collected by dipping the outlet tubing into a 96 well plate containing stop buffer.

3.2.3 Custom-built incubator

In order to keep the reactants at a physiological temperature during the perfusion experiment, it was necessary to enclose the complete system in an incubator. It was not possible to purchase an incubator that would allow the required access to the equipment and so an incubator was custom built in the NIBSC laboratory workshop (see Figure 3.6). The incubator housing was constructed of twin-wall plastic sheets (usually used for greenhouses) and sliding Perspex doors (adapted from a commercial secondary glazing kit). A fan heater connected to a thermocouple was installed in order to regulate the temperature. The thermocouple was mounted on the gantry of the XYZ table at the same level as the flow chamber, ensuring that the chamber was maintained at 37 ± 2 °C.

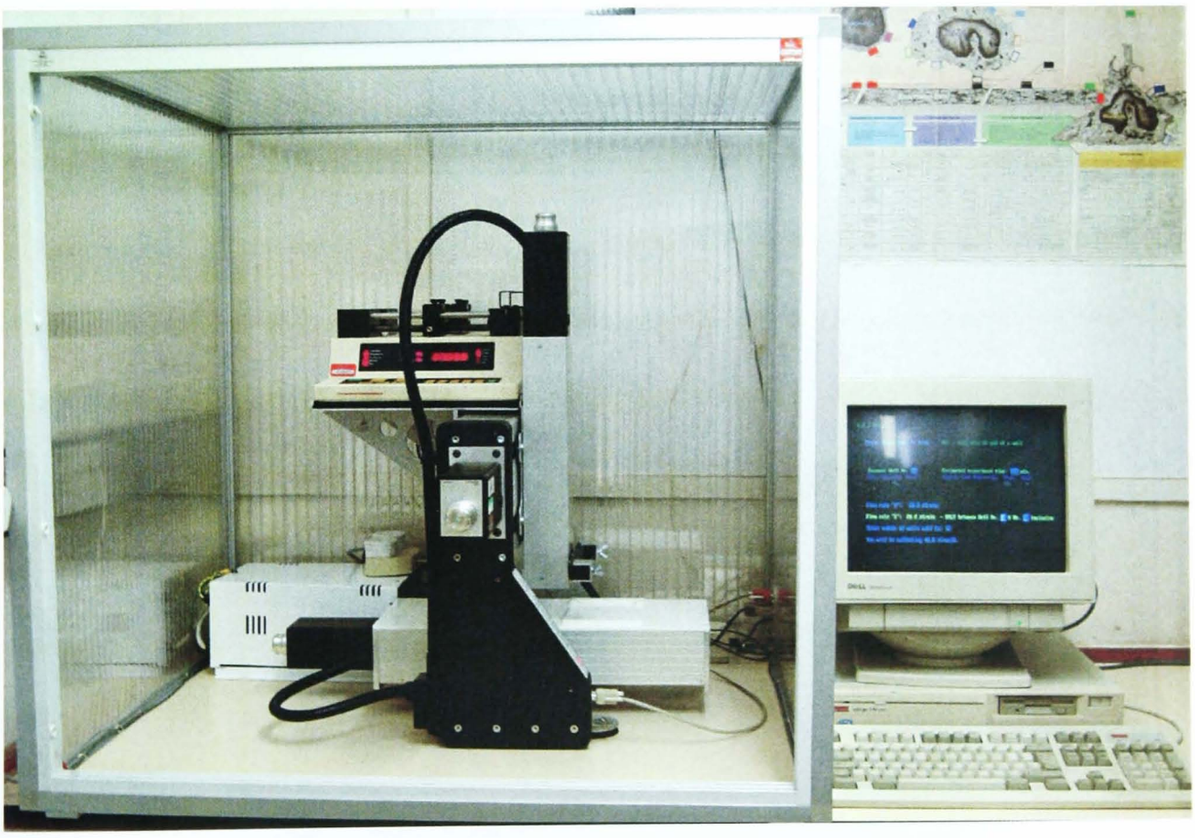


Figure 3.6 *The NIBSC flow system.*
The sliding doors have been removed to aid photography.

3.2.4 Flow chambers

3.2.4.1 Capillary tubing

In order to mimic the *in vivo* environment as accurately as possible, initial work focussed on establishing a protocol for the seeding of endothelial cells on to the inside of polytetrafluoroethylene (PTFE) capillary tubing. The protocol followed that of Lindhout et al (1992), and involved coating the inside of the tubing with 3 $\mu g/cm^2$ fibronectin (Sigma, Poole, UK) at 37 °C for 1 hour and then seeding with a suspension of 0.4×10^6 endothelial cells per cm^2. The tubing was capped and placed inside a modified hybridisation chamber that maintained a temperature of 37 °C and rotated the tubing for up to 3 hours at a rate of 0.5 revolutions per minute, with the aim of attaching an even covering of cells to the inside of the tubing. The tubing was then perfused with growth medium overnight at a low shear rate ($< 10\ s^{-1}$) in order to allow the cells to spread. The cells were then fixed with 2.5% glutaraldehyde and examined by electron microscopy (see Figure 3.7). This method was very labour intensive and not entirely successful, as intact monolayers of cells were not obtained, although cells seeded in this way appeared to resist detachment when the shear rate was increased (up to $67\ s^{-1}$).

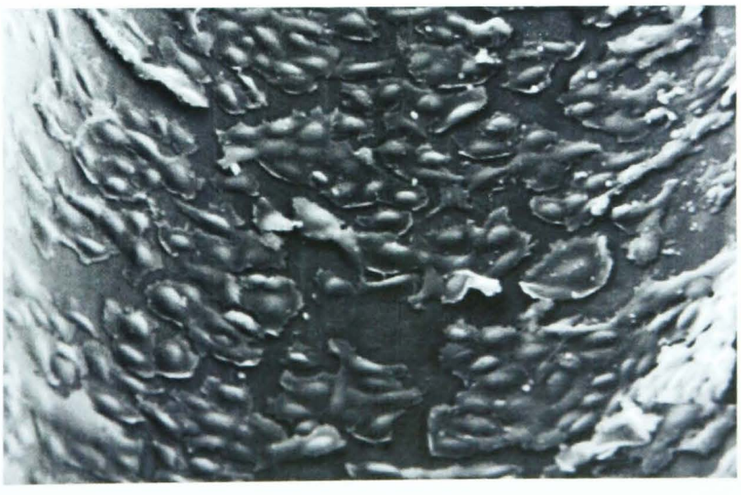


Figure 3.7 *Scanning electron micrograph of endothelial cells lining a PTFE capillary tube.*

3.2.4.2 Parallel plate flow chambers

Two types of parallel plate flow chambers were used in these studies. Both were based on the same principle and are represented by the cross section shown in Figure 3.8.

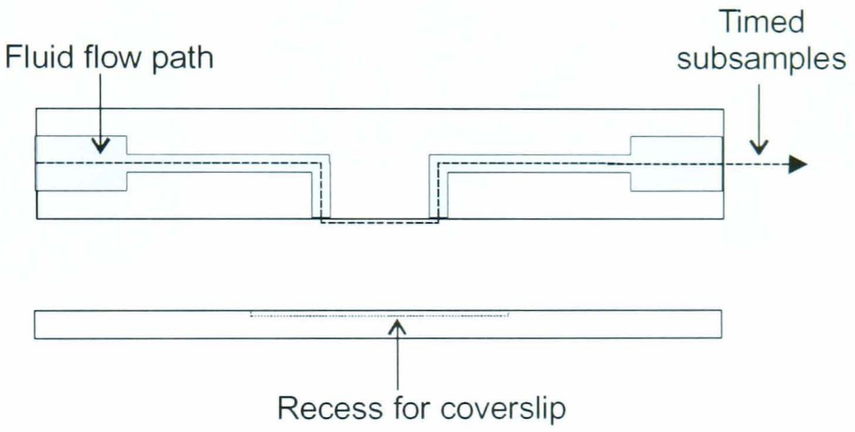


Figure 3.8 *Vertical cross section of a parallel plate flow chamber. The fluid flow path and the position of the coated coverslip are indicated.*

Type 1: This chamber was a gift from Glaxo (Greenford, UK) and was constructed of a Perspex block that screwed to a metal base using wingnuts (see Figure 3.9). The floor of the chamber was formed by the slide portion of a Nunc Slideflask (Life Technologies, Paisley, UK) that fitted into a recess in the metal base. The slit height of the chamber was 150 μm, achieved using a PTFE gasket, and the slit width was 1 cm. Perfusate was pumped through the chamber via holes drilled through the Perspex and nylon cannula tubing (Portex, Hythe, UK). For details of volume of perfusate, flow rates and shear rates, see Table 3.2.

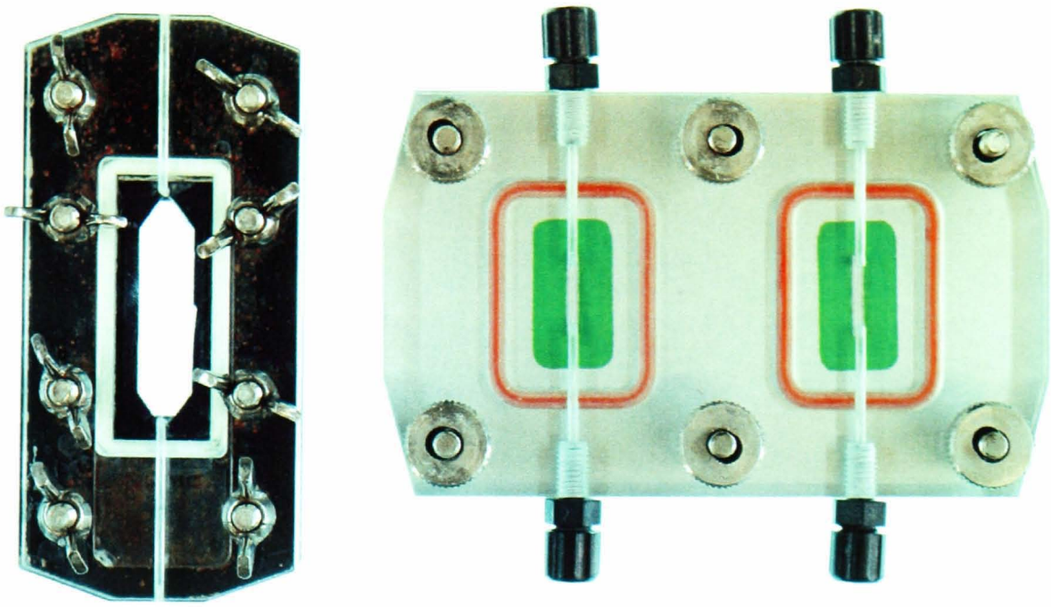


Figure 3.9 *The parallel plate flow chambers*

The Type 1 flow chamber (left), used for studies at the shear rate of 18 s^{-1}, and an example of the Type 2 flow chamber (right), used for studies at shear rates of 178 and 600 s^{-1}.

Type 2: A set of custom-made flow chambers was commissioned from Barnard Engineering (Hertford, UK). A dual-chamber design was decided upon, as this allowed two perfusion experiments to be performed concurrently, saving time and allowing more data to be gathered before the plasma or platelets went beyond their useful lifespan (considered to be 4 hours). Three chambers were manufactured, all with a slit width of 2 mm but with varied slit heights of 50, 75 and 150 µm, achieved using polyester gaskets (see Figure 3.9). Perfusate was pumped through the chamber via holes drilled through the Perspex. PTFE tubing with internal diameter 0.5 mm, designed for use in fast protein liquid chromatography (FPLC), was connected to the chamber using FPLC fittings (Amersham Biosciences, Little Chalfont, UK). For details of volume of perfusate, flow rates and shear rates, see Table 3.2.

Table 3.2 *Summary of chamber dimensions, flow rates and perfusate volumes used in different shear rate experiments.*

Shear rate (s^{-1})	Slit width (cm)	Slit height (μm)	Flow rate (μl/min)	Perfusate volume (μl)	Plasma volume (μl)	Subsample volume (μl)	Number of subsamples
0	-	-	0	1000	800	20	32
18	1.0	150	40	1500	1200	40	32
178	0.2	50	20	1000	800	20	32
600	0.2	75	30	1250	1000	30	32

3.2.5 Software

The software for controlling the flow system was a gift from Dr Yale Nemerson (Mount Sinai Medical Centre, New York, USA) and was modified by Unimatic Engineers (suppliers of the XYZ translation table) to suit the particular model of table and syringe pump chosen. The software enables the user to define flow rates and sampling times so that a wide range of shear rates can be generated and samples of a manageable size collected. The user may also choose to operate one or both syringes for each individual run.

Printouts of the screen displays of the software are found in Appendix 2.

3.3 System validation

Studies were undertaken to assess the suitability of the system for the study of coagulation under flow conditions. These included studies on the effect of flow on the endothelial cell monolayers, the effect of flow on thrombin generation in plasma, the effect of untreated tissue culture plastic on thrombin generation, the effect of shear stress on platelet activation markers and the effect of the presence of red blood cells on the adhesion of platelets.

3.3.1 The effect of flow on endothelial cells

Endothelial cells were prepared on Thermanox coverslips as described in Section 2.9, and were loaded into the flow chamber. Platelet poor plasma was recalcified and perfused through the chamber at a flow rate that generated a wall shear rate of 178 s^{-1} or 600 s^{-1} (equivalent to venous or arterial conditions, respectively) for 32 minutes. After the perfusion, the coverslips were carefully removed from the chamber, washed with TBS and viewed by light microscopy. Micrographs showing some shear-related changes in the morphology but no damage to the monolayer of endothelial cells are shown in Figure 3.10).

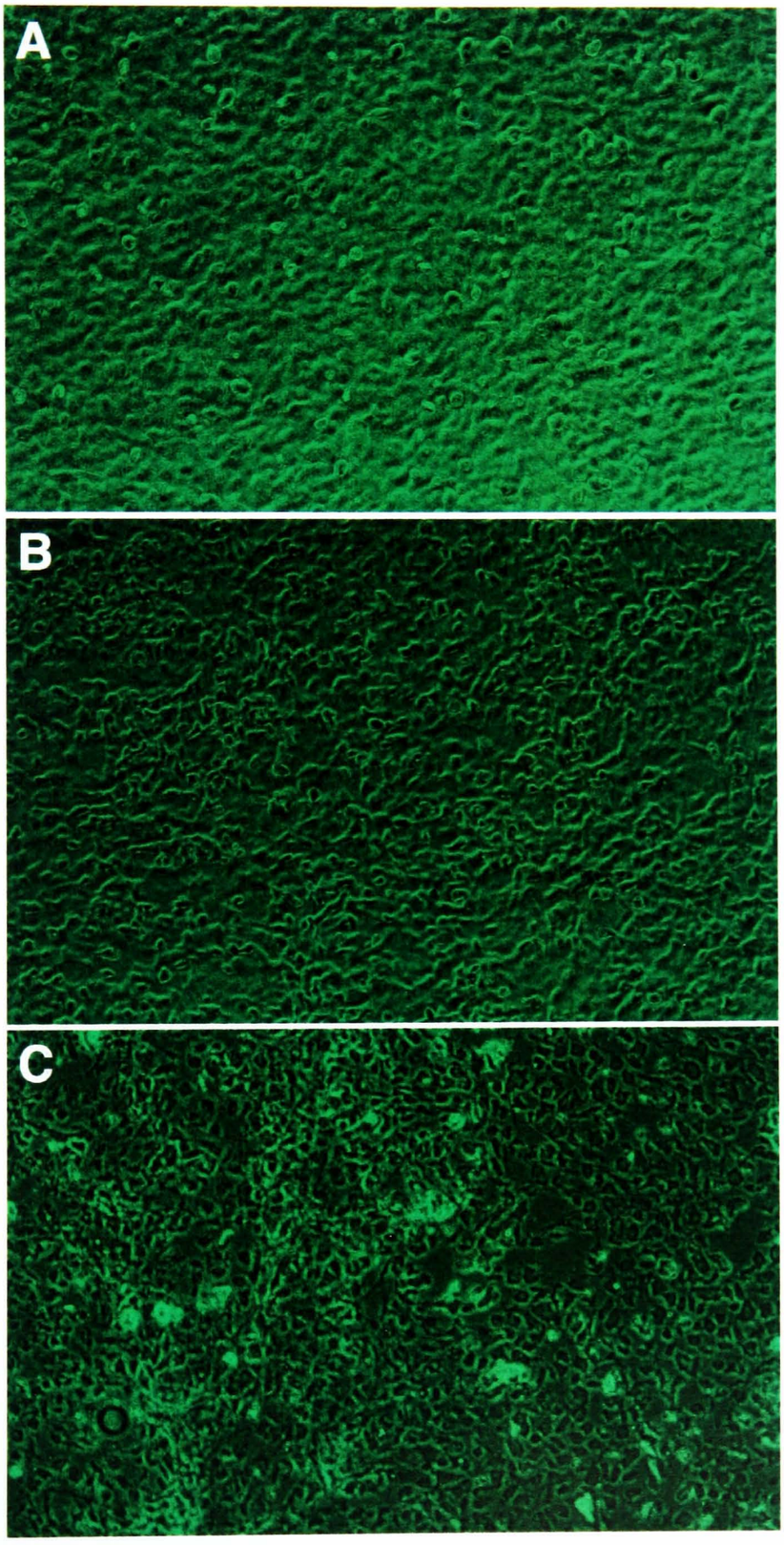


Figure 3.10 *Micrographs of post-perfusion endothelial cells.*

Platelet poor plasma was recalcified and perfused over HUVEC on coverslips mounted in the flow chamber. The coverslips were removed, washed and viewed by light microscopy through a green filter.

A) Before exposure to shear

B) after 32 minutes at 178 s^{-1}

C) after 32 minutes at 600 s^{-1}

3.3.2 The effect of flow on platelets

Platelets may become activated when they are exposed to shear stress and this activation can lead to the release of compounds from intracellular stores and the altered expression of receptors on the platelet membrane. These changes may affect the platelet's contribution to coagulation.

A useful marker of platelet activation is P-selectin, which is an adhesive receptor stored in the α-granules of resting platelets but rapidly relocated to the outer membrane following activation of the platelet (Furie *et al*, 2001). In order to establish the degree of platelet activation that is caused by flow, platelet plasma was prepared as described in Section 2.7 and recalcified with a final concentration of 17.5 mM $CaCl_2$. The plasma was then either pipetted onto a coverslip of HUVEC (static conditions), or perfused through the flow chamber at a shear rate of 600 s^{-1} for 32 minutes (flow conditions). The coverslips were washed, fixed and stained using FITC-anti-P-selectin MAb (1/100 dilution of ascites of Clone Thromb-6, CLB, The Netherlands) and actin and nuclear staining as described in Section 2.11. The cells were examined by confocal fluorescence microscopy, and representative images are shown in Figure 3.11.

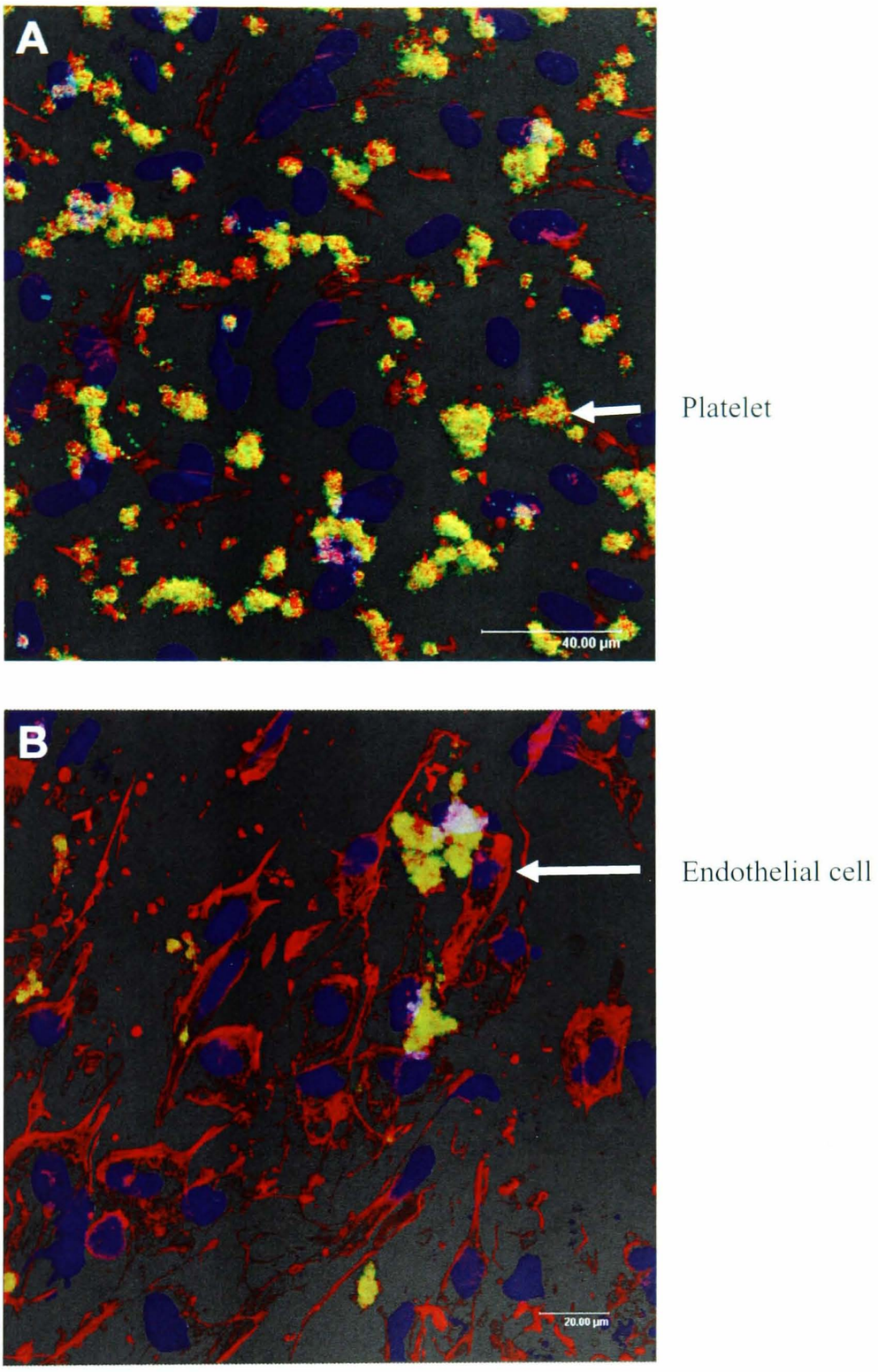


Figure 3.11 *Immunofluorescent staining of endothelial cells and platelets after exposure to static or flow conditions.*

Defibrinated plasma containing 375 x 10^6 platelets/ml was recalcified and A) incubated with HUVEC for 32 minutes, or B) perfused at 600 s^{-1} across HUVEC in a flow chamber for 32 minutes. The cells were then fixed with 2 % paraformaldehyde, incubated with FITC anti-P selectin monoclonal antibody Thromb 6 (green) for 30 minutes followed by 66 nM Alexa Fluor 532 phalloidin (to stain actin red) in PBS containing 1 % Triton X-100. Nuclei are stained blue following a 10 minute incubation with Hoechst 33342.

3.3.3 The thrombin generation test

Background information and details of the materials and methods used in the thrombin generation tests are described in Section 2.8. Briefly, normal pooled plasma was defibrinated with ancrod and fresh platelets (obtained from a group O donor by centrifugation of platelet rich plasma) were resuspended in this plasma at a physiological concentration to give 'platelet plasma'. Coagulation was initiated by the addition of an activator such as tissue factor and calcium chloride (TF/Ca^{2+}).

3.3.3.1 The effect of flow on thrombin generation in platelet poor plasma

When defibrinated normal plasma without platelets was triggered with TF/Ca^{2+} and pumped through the flow chamber at a shear rate of 18 s^{-1} with an untreated plastic slide forming the base of the chamber, significantly more thrombin was generated than under static conditions (AUC of 2666 ± 694 and 1206 ± 170 IU.seconds/ml respectively; mean ± sd; $n \geq 8$; $p < 0.01$). When the plastic slide had been pre-treated for 1 hour at 37 °C with a 1 % (v/v) solution of HSA (BPL, Elstree, UK) in TBS, significantly less thrombin generation was seen (AUC of 766 ± 202 IU.seconds/ml, $n = 3$; $p < 0.01$), suggesting that the negative charge on the tissue culture treated plastic provides a favourable surface for coagulation.

To investigate this hypothesis, the non-activated partial thromboplastin time (NAPTT) assay was performed on defibrinated plasma that had been passed over an untreated plastic slide at a shear rate of 18 s^{-1}. The NAPTT assay is highly sensitive to the presence of activated clotting factors, in particular FIXa (Prowse & Pepper, 1980). The plasma was mixed in a 1:1 ratio with a normal plasma (in order to provide enough fibrinogen for a detectable clot to form), and 100 µl of this mix was

added to 100 µl of 50 µg/ml bovine brain phospholipid reagent (NIBSC reagent 91/542, diluted in TBS) and 100 µl of 25 mM $CaCl_2$ in a plastic tube. The tubes were incubated at 37 °C and the time taken for the mixture to clot (by visual determination) was recorded. The plasma that had been passed through the flow chamber clotted in 260 ± 8 seconds (mean of 8 samples, taken at 3 minute intervals during the 24 minute perfusion) compared to 299 ± 7 seconds (n = 2) for the control plasma. This shortening of the clotting time ($p < 0.001$) suggests that there may be some activation of components of the intrinsic coagulation pathway during the perfusion of the plasma through the flow chamber. It is therefore possible that negative charges found on the plastic slide or in the tubing may be facilitating the activation of FXI, leading in turn to the activation of FIX.

3.3.3.2 The effect of platelets on thrombin generation

The presence of platelets in the reaction mixture resulted in significantly more thrombin generation than in their absence. The platelet count that was required for maximal thrombin generation was studied in a series of single experiments using plastic tubes and static conditions and either HUVEC or ECM at venous or arterial shear rates (see Figure 3.12).

Fig 3.12a

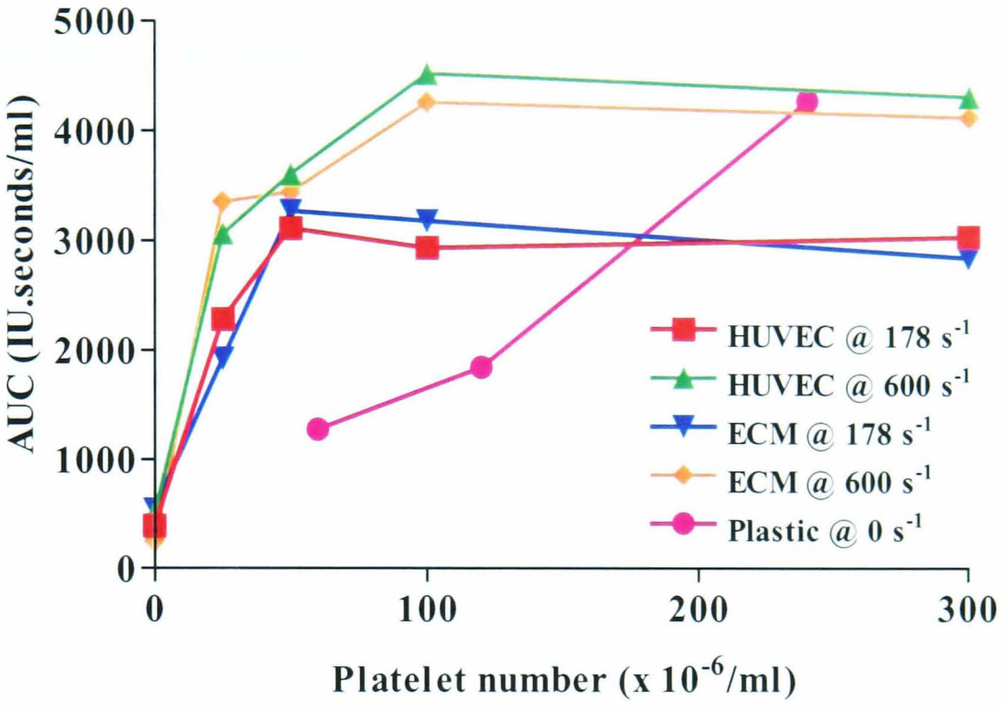


Fig 3.12b

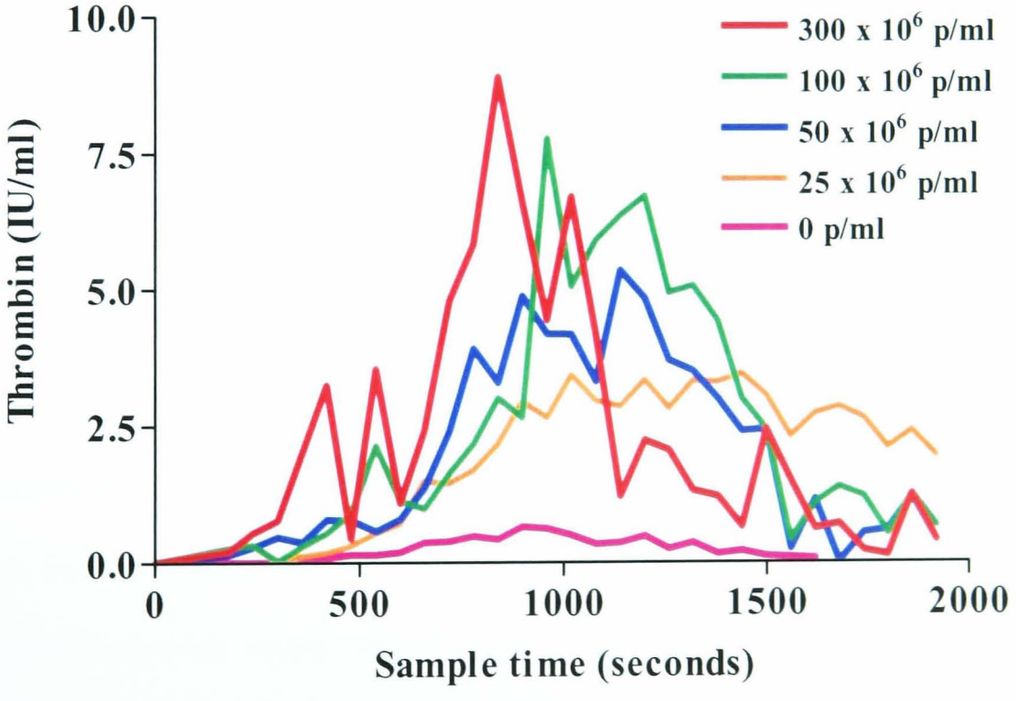


Figure 3.12 *The effect of platelet concentration on thrombin generation.*

Thrombin generation was initiated in defibrinated plasma containing platelets using 35 pM TF and calcium. a) Dose response curves under different shear conditions and in the presence of HUVEC or ECM and 300 x 10^6 platelets/ml; b) thrombin generation curves from the platelet dose response experiment over ECM at 600 s^{-1}.

Under static conditions a dose response to platelet count was seen up to 240 x 10^6 platelets/ml ('Plastic @ 0 s^{-1}' in Figure 3.12a), the highest concentration of platelets tested, and this is similar to the results of Reverter *et al* (1996) who found that 300 x 10^6 platelets/ml were required for near maximal thrombin generation under static conditions following similar activation with TF/Ca^{2+}. The contribution of platelets is not surprising as they carry coagulation factors within and on the surface of their membrane and, once activated, they also provide a negatively charged phospholipid surface that acts as a catalytic template for the interaction of coagulation factors. Under flow conditions maximal thrombin generation was seen at 50 x 10^6 platelets/ml at a shear rate of 178 s^{-1} and 100 x 10^6 platelets/ml at 600 s^{-1}, with no further increase up to 300 x 10^6 platelets/ml (Figure 3.12a). In these single experiments, the maximum thrombin generation appeared to be higher at 600 s^{-1} than at 178 s^{-1}, but the difference between these shear rates was not significant when further experiments were performed (see Figure 3.13). In order to achieve maximal thrombin generation in the static system a final platelet concentration of 300 x 10^6 per ml was chosen for subsequent experiments. For direct comparability with the static system, this concentration was also used for the flow experiments.

Examples of the thrombin generation curves obtained with different platelet counts under flow conditions are shown in Figure 3.12b. An example of the effect of platelets on absolute values of AUC, peak thrombin and time of peak thrombin from a series of experiments using ECM and a shear rate of 178 s^{-1} are shown in Table 3.3.

Table 3.3 *To illustrate the effect of platelets on absolute values of AUC, peak thrombin and time of peak thrombin.*

Data are from ECM experiments at 178 s^{-1} with no inhibitor, with or without 300 x 10^6 platelets/ml and following initiation of thrombin generation with 35 pM TF and calcium. Data are expressed as mean ± standard error of mean, n ≥ 2.

	AUC (IU.seconds/ml)		Peak (IU/ml)		Peak time (seconds)	
γ (s^{-1})	0	178	0	178	0	178
- platelets	512 ± 174	617 ± 42	0.5 ± 0.2	1.3 ± 0.2	1140 ± 300	830 ± 61
+ platelets	3196 ± 134	3324 ± 164	5.2 ± 0.3	5.3 ± 0.4	967 ± 37	1000 ± 47

3.3.3.3 The effect of tissue culture plastic on thrombin generation in platelet plasma

Platelet plasma was triggered with TF/Ca^{2+} and pumped through the flow chamber at a shear rate of 18 s^{-1} with either an untreated plastic slide or a plastic slide pre-treated with a 1 % (v/v) solution of HSA (BPL, Elstree, UK) in TBS forming the base of the chamber. Pre-treatment with albumin resulted in a small but significant reduction in the amount of thrombin generated from 3915 ± 796 to 3661 ± 797 IU.seconds/ml respectively (mean ± sd; n ≥ 5). The albumin pre-treatment was intended to block non-specific adsorption of plasma proteins to the slide, and the result suggests that the thrombin generation seen is not dependent upon adhesion of platelets to the adsorbed plasma proteins, leading to surface-bound coagulation reactions, but is also occurring on platelets in the fluid phase.

3.3.3.4 The effect of HUVEC or ECM on thrombin generation in platelet plasma

Under static conditions significantly less thrombin was generated in platelet plasma in the presence of HUVEC than ECM (2582 ± 216 and 3196 ± 410 IU.seconds/ml respectively; n = 9; $p < 0.001$; Figure 3.13). At a shear rate of 178 s^{-1} thrombin generation was significantly increased in the presence of HUVEC to 3132 ± 361 IU.seconds/ml (n = 16; $p < 0.001$) but no further increase was seen in the presence of ECM, which remained at 3238 ± 609 IU.seconds/ml.

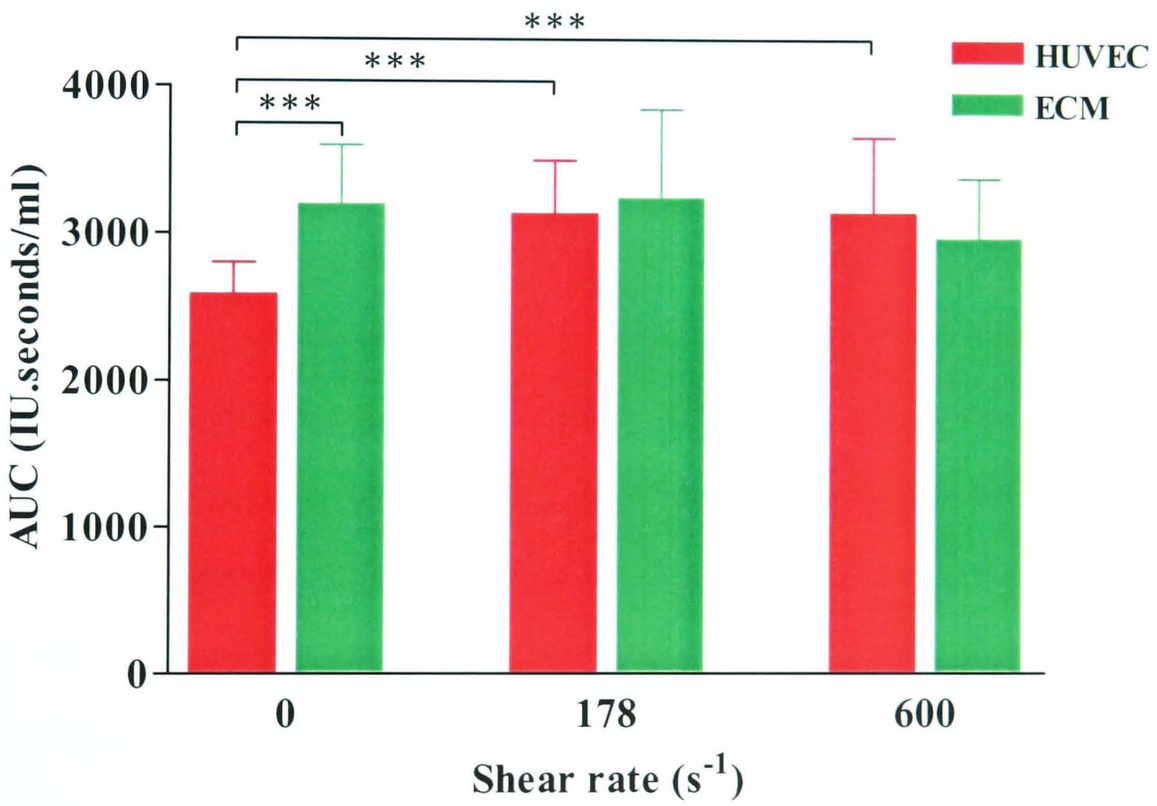


Figure 3.13 *Total thrombin generation at static, venous or arterial shear rates in the presence of HUVEC or ECM.*

*Thrombin generation was initiated with 35 pM TF and calcium in defibrinated plasma containing 300 x 10^6 platelets/ml. At shear rate 0 s^{-1}, the activated plasma was incubated in a culture well containing HUVEC or ECM and for 178 and 600 s^{-1} the activated plasma was perfused through a parallel plate flow chamber containing HUVEC or ECM. Mean ± standard deviation; n ≥ 9; ***p < 0.001.*

No further significant changes were seen in thrombin generation when the shear rate was increased to 600 s^{-1}, with thrombin generation of 3135 ± 521 and 2967 ± 410 IU.seconds/ml being seen in the presence of HUVEC and ECM respectively (n = 11). This suggests that although intact HUVEC are less thrombogenic than ECM under static conditions, they lose some of their antithrombotic character when exposed to shear stress.

3.3.4 The effect of the presence of red blood cells

Red blood cells may increase the rate of coagulation by releasing the platelet agonist ADP (Gaarder *et al*, 1966), and by providing negatively charged phospholipid membranes for the assembly of coagulation factor complexes (Peyrou *et al*, 1999). Platelet adhesion under flow conditions is also affected by the presence of red blood cells, as they occupy the central region around the axis of the vessel (Sakariassen *et al*, 2001). This forces the platelets nearer to the vessel wall, where they are well placed to become activated and/or adherent following the interaction of their receptors with ligands exposed in the sub-endothelium. Experiments were performed in order to test whether this process could have an effect in the thrombin generation test under static and flow conditions, where red cells cannot be included due to their interference with the chromogenic determination of thrombin concentration.

Washed red blood cells were prepared following the removal of PRP from whole blood, after centrifugation (150 g for 10 minutes). The red blood cells were diluted with an equal volume of PBS-A (0.17 M NaCl, 3.35 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4; pH 7.4) with 5 mM glucose (all reagents from BDH, Poole, UK) and centrifuged at 1000 g for 5 minutes. The supernatant was discarded and the packed cells resuspended in PBS + glucose, centrifuged at 2000 g for 5 minutes and

the process repeated with a final spin of 2000 g for 15 minutes. The supernatant was discarded and the packed red blood cells were counted using a Coulter AcT8 Cell Counter in pre-dilution mode. This method gave a haematocrit between 80 and 90 % (Lankhof *et al*, 1995).

A reaction mixture was prepared with 40 % (v/v) packed red blood cells, 40 % platelet plasma (750 x 10^6 platelets/ml) and 2.5 % TBS. The mixture was activated by the addition of 17.5 % low TF/Ca^{2+} and perfused over HUVEC or ECM at 178 s^{-1} and 600 s^{-1} as described in Section 2.8.3. For static conditions, a variation from the method described in Section 2.8.2 was used, with an ECM-coated coverslip being placed in a culture well. Following the perfusion, the coverslips were fixed with 0.5 % glutaraldehyde in PBS-A for 15 minutes and dehydrated in methanol for 15 minutes prior to staining with a 1:1 mixture of May-Grunwald and Giemsa stains (Sigma, Poole, UK) for 5 minutes. This mixture contains eosin, which stains the cytoplasm and acidic granules red, and azure blue, which stains nuclear material. After staining, the coverslips were mounted and viewed by light microscopy. Photomicrographs are shown in Figure 3.14.

The effect of the increased dilution of plasma on thrombin generation, which could have a significant effect on the activation of platelets, was checked using 40 % (v/v) TBS in place of packed red blood cells. The extra dilution resulted in a 20 % decrease in thrombin generation (4476 and 3620 IU.seconds/ml with 80 and 40 % plasma respectively). It has previously been reported that a reaction mixture containing red blood cells in platelet poor plasma may support 40 % more thrombin generation than platelet rich plasma, with this difference suggested to be due to the

enhanced inactivation of FV by Protein C on platelet membrane phospholipids (Tans *et al*, 1991; Peyrou *et al*, 1999).

The presence of red blood cells did not appear to have an effect on platelet adhesion under static conditions (Figure 3.14 A and B). At 178 s^{-1} more platelets appeared to adhere to ECM in the absence of red blood cells than in their presence (Figure 3.14 C and D). The opposite was true at 600 s^{-1}, where a clear difference may be seen with large aggregates forming in the presence of red blood cells (Figure 3.14 E and F). Platelet adhesion to HUVEC may be seen following perfusion at 600 s^{-1} both in the presence and absence of red blood cells (Figure 3.15), although larger aggregates appear in the presence of red blood cells.

These results suggest that red blood cells have an effect on platelet adhesion under flow conditions. At the venous shear rate of 178 s^{-1}, the reduced adhesion of platelets in the presence of red blood cells may be the result of less segregation of the cell types in the flowing perfusate, leading to reduced platelet-ECM interaction due to the presence of red blood cells in the boundary layer. Increased adhesion in the presence of red blood cells at the arterial shear rate 600 s^{-1} suggests that segregation of the cell types may be occurring in the perfusate and that platelets are indeed forced closer to the coverslip, where they adhere to the adhesive proteins in the ECM and also to HUVEC. This is in agreement with published theories on blood flow (reviewed by Sakariassen *et al*, 2001), and indicate that the presence of red blood cells is desirable in studies of platelet adhesion and thrombin generation under flow conditions. The absence of red blood cells from these studies, due to their

interference in the detection of thrombin, is a limitation of this study and a challenge to be addressed.

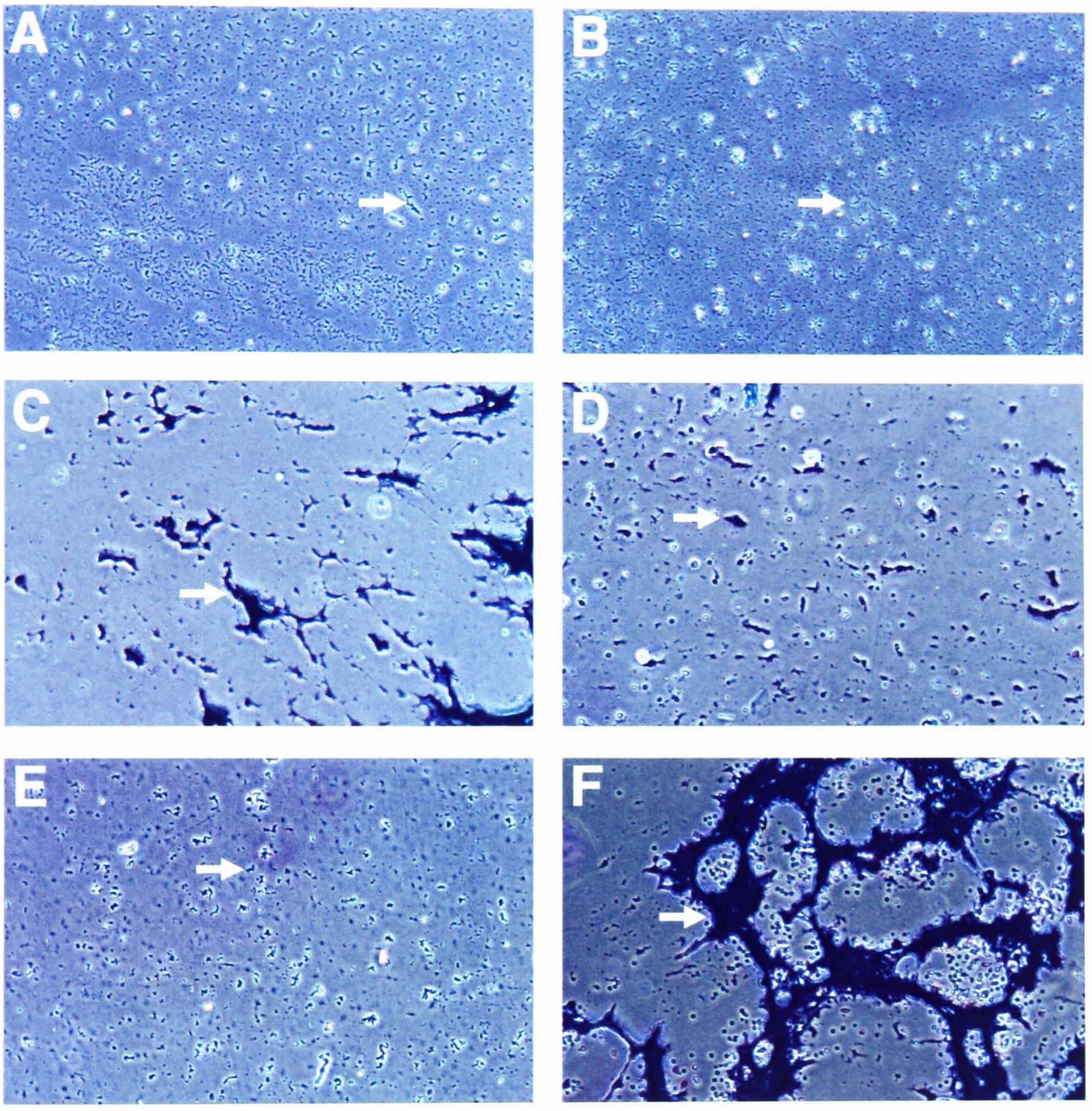


Figure 3.14 *Photomicrographs of platelet adhesion to ECM in the absence or presence of red blood cells.*

A reaction mixture was prepared with 40 % (v/v) packed red blood cells, 40 % platelet plasma (750 x 10^6 platelets/ml) and 2.5 % TBS. The mixture was activated by the addition of 17.5 % low TF/Ca^{2+} (final concentration of TF = 35 pM) and perfused over ECM at 178 s^{-1} and 600 s^{-1}. For static conditions, an ECM-coated coverslip was placed in a culture well. Following perfusion the cells were fixed with 0.5 % glutaraldehyde and stained with mixed May-Grunwald and Giemsa stains. Adherent platelets and platelet aggregates are indicated by arrows.

A) 0 s^{-1} – RBC

B) 0 s^{-1} + RBC

C) 178 s^{-1} – RBC

D) 178 s^{-1} + RBC

E) 600 s^{-1} – RBC

F) 600 s^{-1} + RBC.

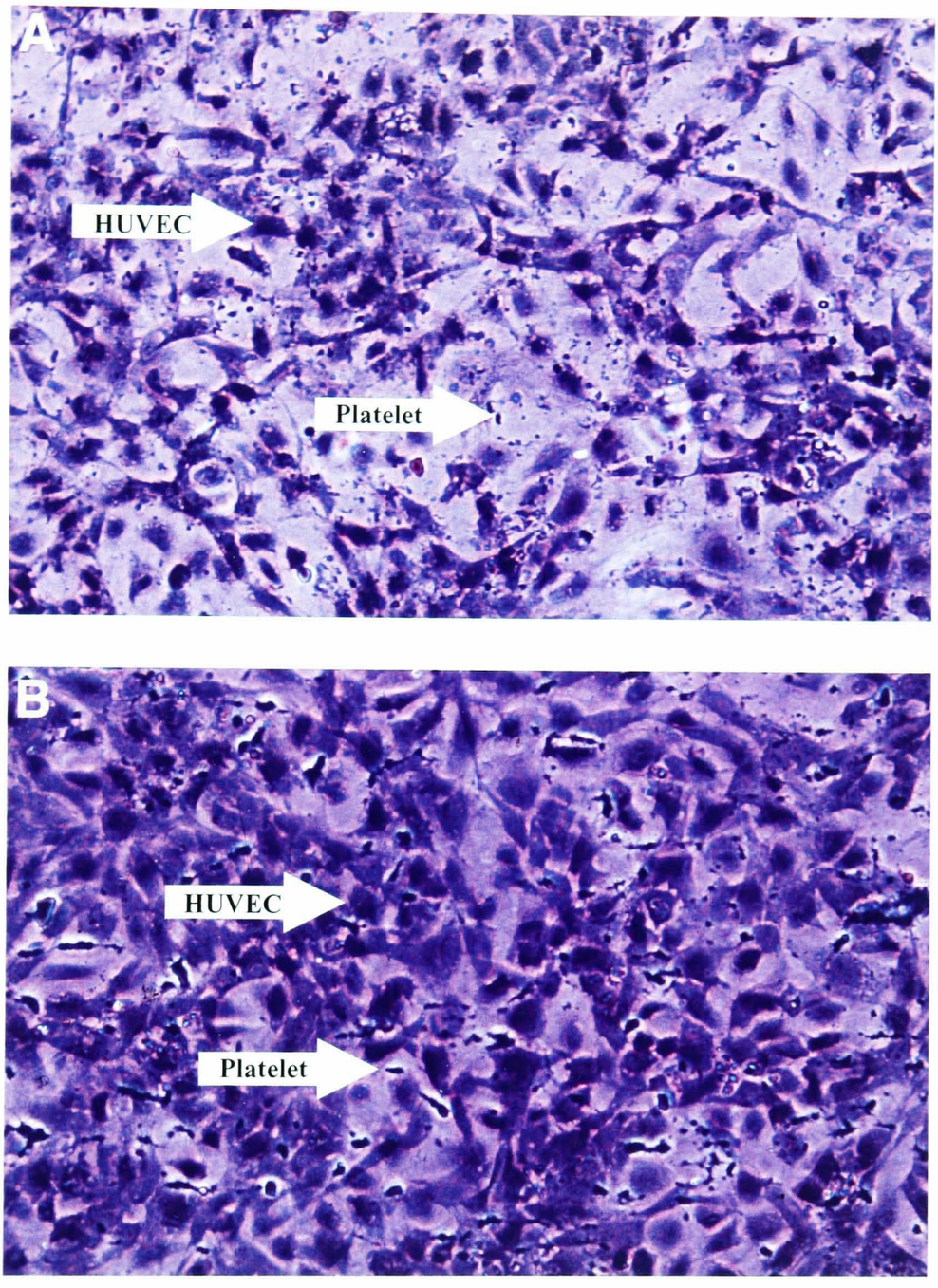


Figure 3.15 *Platelet adhesion to HUVEC in the absence or presence of red blood cells.*

A reaction mixture was prepared with 40 % (v/v) packed red blood cells, 40 % platelet plasma (750 x 10^6 platelets/ml) and 2.5 % TBS. The mixture was activated by the addition of 17.5 % low TF/Ca^{2+} (final concentration of TF = 35 pM) and perfused over HUVEC at 600 s^{-1}. Following perfusion the cells were fixed with 0.5 % glutaraldehyde and stained with mixed May-Grunwald and Giemsa stains. Adherent platelets are indicated by arrows. Following perfusion the cells were fixed with 0.5 % glutaraldehyde and stained with mixed May-Grunwald and Giemsa stains. HUVEC and adherent platelets are indicated with arrows.

A) 600 s^{-1} – RBC

B) 600 s^{-1} + RBC

3.4 Discussion

The effects of flow on the process of blood coagulation are numerous. Physical effects on vessel wall components such as endothelial cells and their extracellular matrix may be observed, and platelets circulating in the blood may become primed for adhesion and activation. By increasing the rate of diffusion, flow increases the rate of interaction of fluid phase substrates and vessel wall-bound enzymes, facilitating coagulation. When designing an assay system to study coagulation, it is therefore important that all these components are taken into consideration.

In designing the NIBSC flow system, consideration was given to the inclusion of platelets and endothelial cells and to the range of *in vivo* flow conditions that could be modelled. A parallel plate design was the most suitable for the inclusion of endothelial cells and their removal for examination after completion of the experiment. The dimensions of the chambers to be used were calculated to give physiologically relevant shear rates without requiring a large volume of plasma, as this was usually the limiting factor. Collection of timed subsamples at the outlet of the chamber was easily achieved and the use of an EDTA-containing stop buffer allowed the thrombin concentration of each subsample to be determined at the end of each run.

The shear rates studied were similar to those found in static, venous and arterial conditions, and these were seen to have some physical effects on both endothelial cells and platelets. The endothelial cells appear unaffected by shear stress when examined by light microscopy, but immunofluorescent examination revealed changes to the actin cytoskeleton that appeared to align with the direction of flow. The

activation marker P-selectin was not detected on the surface of the endothelial cells, suggesting that they were not activated by shear stress. Platelets adhered to endothelial cells regardless of the presence of flow, but larger aggregates were seen after perfusion than after static incubation. P-selectin was detected on the surface of platelets in both cases and may be the result of platelet activation by small amounts of thrombin generated in the recalcified plasma.

Coagulation, as determined by measurement of thrombin generation, was seen to increase under flow conditions and to increase dramatically in the presence of platelets. Coagulation was seen to be occurring in the fluid phase and not to be restricted to the vicinity of the vessel wall, as coagulation still occurred when the vessel wall was pre-treated with albumin. However, the vessel wall did appear to have an influence under static conditions where significantly less thrombin was generated in the presence of intact endothelium than extracellular matrix. The absence of red blood cells in the perfusate is a limitation of the system that may reduce platelet interaction with endothelial cells or extracellular matrix, but was a necessary omission to enable the detection of thrombin.

The NIBSC flow system is a useful device for the study of coagulation in the presence of the highly relevant factors such as shear stress, vessel wall components and platelets. The study of inhibitors of coagulation in this system may give *in vitro* data that is more pertinent to the *in vivo* situation than more conventional approaches.

CHAPTER 4

ANTI-PLATELET ANTIBODIES

4.1 Introduction

Platelets play a critical role in primary and secondary haemostasis, and they are therefore potential targets for antithrombotic agents. Inhibition of platelet function by aspirin is effective but non-specific, and inhibitors of platelet ADP receptors (such as clopidogrel) are under investigation as specific anti-platelet agents, as discussed in Section 1.3.7.4. Direct inhibition of the adhesive and aggregatory receptors and the inhibition of coagulation factor interaction on the platelet membrane are therefore alternative routes by which the contribution of platelets to coagulation may be specifically modulated.

4.1.1 Inhibitors of GP IIb/IIIa

Antagonists of GP IIb/IIIa, the main aggregatory receptor on the platelet membrane, will clearly inhibit the ability of activated platelets to aggregate. In addition to this effect, *in vitro* studies have shown that antibodies and peptide and non-peptide antagonists of GP IIb/IIIa can partially inhibit the generation of thrombin in a platelet dependent system, indicating that anti-platelet agents interfere with coagulation. (Reverter *et al*, 1996; Pedicord *et al*, 1998; Herault *et al*, 1998; Butenas *et al*, 2001). There is evidence that anti-GP IIb/IIIa agents inhibit the exposure of negatively charged phospholipids on the platelet membrane (Pedicord *et al*, 1998). GP IIb/IIIa antibodies have also been reported to interfere with the shape change seen in activated platelets and it is possible that this may limit the activation of the platelet and the scrambling of the membrane (Isenberg *et al*, 1990). This would contribute to their effectiveness as antithrombotic drugs, as in addition to preventing the cross-linking of platelets at sites of injury they may also be able to inhibit the coagulation

cascade. It has also been reported that prothrombin binds to resting GP IIb/IIIa on platelets (Byzova & Plow, 1997) where it is held in close proximity to the prothrombinase complex that assembles on the membrane, thus facilitating prothrombin conversion. An agent with higher affinity for GP IIb/IIIa may displace prothrombin from its convenient position and reduce the rate of conversion.

4.1.2 Inhibitors of GP Ib/IX/V

The main function of the GP Ib/IX/V complex is to mediate adhesion of the platelet to areas of damage to the vessel wall, as discussed in Section 1.2.1.1, and interference with this process will therefore inhibit primary haemostasis. The GP Ib/IX/V complex also functions as a high affinity receptor for thrombin (Harmon & Jamieson, 1986; Harmon & Jamieson, 1988; Dong *et al*, 1997) and inhibition of the complex may interfere with platelet activation (De Marco *et al*, 1991; Yamamoto *et al*, 1991) and therefore have an effect on secondary haemostasis. A link between fibrin, vWF and GPIbα has also been demonstrated to play a role in platelet dependent thrombin generation (Béguin *et al*, 1999). In addition, the GP Ib/IX/V complex has been shown to be a high affinity binding site for FXI, promoting its activation by thrombin and therefore potentiating intrinsic activation of coagulation (Baglia *et al*, 2002).

4.1.3 Studies undertaken

Platelet aggregometry was used as a screening test to ensure that the antibodies used were indeed platelet inhibitors and to assess the effective concentrations to be studied further in other assay systems. The thrombin generation test was chosen as the most

appropriate method for the analysis of coagulation for reasons detailed in Section 2.8. Studies were performed using intrinsic stimulation and extrinsic stimulation with either high (630 pM) or low (35 pM) TF under static conditions using the clotting method for thrombin detection. When this section of work was begun, the software required for analysis of chromogenic thrombin detection data was not yet available. Following the studies using intrinsic and high TF extrinsic stimulation, and those of others (van't Veer & Mann, 1997; Cawthern *et al*, 1998; Keularts *et al*, 2001; Butenas *et al*, 2001), it was decided to proceed with low TF extrinsic stimulation as the most physiologically relevant method, and studies began using the Type 1 flow chamber (see Section 3.2.4.2), using the chromogenic method of thrombin detection. It was only possible to generate a relatively low shear rate in the Type 1 flow chamber, so the Type 2 chambers were designed and commissioned, enabling studies at higher shear rates without excessive consumption of reagents.

The inhibitory effects of the anti-platelet antibodies that were detected in the thrombin generation tests were analysed further. Flow cytometry was used to examine the effect of the antibodies on the exposure of negatively charged phospholipids on the surface of the platelet membrane. Assays to detect the presence of soluble fibrin in defibrinated plasma and the inclusion of an anti-vWF antibody in thrombin generation tests were used to examine the influence of the fibrin-vWF-GP Ibα pathway on thrombin generation.

4.2 Materials and Methods

4.2.1 Antibodies

RFGP56 is a murine monoclonal IgG_1 that binds to a complex-specific epitope on GP IIb/IIIa. It does not bind to platelets from patients with Glanzmann's Thrombasthenia (GT) or to EDTA-treated platelets, but it does bind to platelets from patients with Bernard-Soulier Syndrome (BSS). It totally inhibits platelet aggregatory responses to thrombin, collagen, ADP, arachidonate and calcium ionophore A23187. It also inhibits the binding of fibrinogen and vWF to platelets (Cox, 1991). The antibody was produced by Cymbus Biotechnology (Southampton, UK) under contract from the owner of the hybridoma, Dr Alison H. Goodall (University of Leicester, UK).

RFGP37 is a murine monoclonal IgG_1 that binds to GP Ibα. It binds to platelets from patients with GT but does not bind to platelets from patients with BSS. It completely inhibits ristocetin-induced platelet agglutination and adhesion of platelets to vWF under conditions of high shear (Cox, 1991). The antibody was produced by Cymbus Biotechnology (Southampton, UK) under contract from the owner of the hybridoma, Dr Alison H. Goodall. A single production batch was used for all experiments at 0 and 18 s^{-1} and some experiments at 178 and 600 s^{-1}. Subsequent batches gave conflicting results in routine platelet aggregometry tests, and investigations by Dr Goodall led to the conclusion that the batches were contaminated with another antibody, possibly RFGP56. Serotec (Oxford, UK), another biotechnology company, was then contracted to produce fresh antibody from hybridoma cells supplied by Dr Goodall. This antibody performed as expected in platelet aggregometry, in that it inhibited ristocetin-induced agglutination, and flow

cytometry in Dr Goodall's laboratory. A full set of thrombin generation experiments were therefore performed at 178 and 600 s^{-1}. Due to the different properties of RFGP37 produced by Cymbus or Serotec, the company name is reported alongside the antibody name in the results section. In addition, a small quantity of RFGP37 (batch ST0802) was purified from supernatant harvested from cultures of the hybridoma in 1994 and stored at –80 °C. Antibody from 25 ml of the supernatant was captured on a 1 ml Protein G column (Amersham Biosciences, Chalfont St Giles, UK), eluted at pH 2.7, and dialysed into TBS.

c7E3 was a gift from Centocor Ltd, Malvern, USA. It is a chimeric Fab fragment, with mouse variable regions and human constant regions (Centocor commercial information), of a murine IgG immunoglobulin against GP IIb/IIIa and $\alpha_V\beta_3$ (Coller *et al*, 1983).

Reg A, a MAb against the Rhesus D antigen on red blood cells (Bye *et al*, 1992), was used as a negative control in platelet aggregation and flow cytometry assays. It was a gift from Dr Willem Ouwehand of the Division of Transfusion Medicine, University of Cambridge, UK.

RFFVIII:R/1, a monoclonal antibody against the GP Ibα binding site on vWF, was a gift from Dr Alison H Goodall of the Division of Clinical Biochemistry, University of Leicester, UK. The ammonium sulphate cut of ascites (> 9 mg/ml) was diluted in TBS before use.

4.2.2 Platelet aggregation

Platelet aggregation assays were performed on a BioData PAP-4 Aggregometer (Alpha Laboratories, Eastleigh, UK). A platelet pellet was prepared as described in Section 2.7 and resuspended in 1 ml of TSHG buffer, which comprised 0.05 M Tris(hydroxymethyl) aminomethane 0.15 M NaCl, 5 mM glucose (BDH reagents from Merck, Poole, UK) and 0.5 % v/v HSA (Zenalb, BPL, Elstree, UK) adjusted to pH 7.4). The platelet count was adjusted to a final concentration of 250 x 10^6 per ml and 178 µl of the platelet suspension and 22 µl of either TBS or antibody (up to 180 µg/ml in TBS) were incubated at 37 °C with stirring at 1000 rpm for 5 minutes. Aggregation was stimulated with 20 µl of human α-thrombin solution (final concentration 0.05 – 0.5 IU/ml; NIBSC reagent 94/708 diluted in TBS + 1% HSA). Results were calculated from the aggregation seen 5 minutes after the addition of thrombin and aggregation seen in the absence of any antibody (TBS alone) was taken to be 100%.

4.2.3 ELISA to evaluate specificity and inhibition of thrombin binding of anti-GP Ibα antibodies

These assays were performed by Mr Hans Ulrichts, University of Leuven, Kortrijk, Belgium. Unless otherwise stated, the reagents used were from Sigma, Bornem, Belgium and the monoclonal antibodies and recombinant proteins were made in house.

4.2.3.1 To evaluate the specificity of anti-GP Ibα monoclonal antibodies

Ninety-six well microtiter plates (Greiner, Frickenhausen, Germany) were coated overnight at 4 °C with the antibodies (5 µg/ml in PBS), blocked with 250 µl/well of TBS + 3 % milk powder and incubated for 2 hours at 37°C with a dilution series of recombinant GP $Ib\alpha_{1-289}$ in TBS + 0.1% Tween-20 (TBST), starting from 2.5 µg/ml. Wells were washed 12 times with TBST, and bound recombinant GP $Ib\alpha_{1-289}$ was detected with 1 µg/ml of biotinylated anti-GP Ibα MAbs 6B4 and 12G1 for 90 minutes at room temperature. Bound biotinylated anti-GP Ibα MAb 6B4 and MAb 12G1 were detected with streptavidin conjugated horseradish peroxidase for 45 minutes at room temperature. Visualisation was obtained with ortho-phenylenediamine and the colour reaction was stopped with 4 M H_2SO_4 after which the absorbance was determined at 490 nm.

4.2.3.2 To evaluate inhibition of thrombin binding to recombinant GP Ibα.

Wells were coated with 5 µg/ml of anti-GP Ibα MAb 12G1 overnight at 4 °C and blocked for 2 hours at room temperature with 1% bovine serum albumin. After washing, wells were incubated for 60 minutes at 4 °C with 1 µg/ml recombinant GP Ibα. Wells were washed and preincubated with a dilution series of the anti-GP Ibα MAbs, the positive control MAb 2D2 and negative control MAb 16G5. After 30 minutes at 4 °C a constant amount of α-thrombin was added (0.75 µM final concentration). After 60 minutes incubation at 4 °C, wells were washed and residual bound α-thrombin was detected with the chromogenic substrate S-2238 (Chromogenix-Instrumentation Laboratory, Milan, Italy).

4.2.3.3 To evaluate inhibition of thrombin binding to glycocalicin

Wells were coated with 2 µg/ml recombinant glycocalicin overnight at 4 °C and blocked for 2 hours at room temperature with 1% bovine serum albumin. After washing, wells were preincubated with a dilution series of the anti-GP Ibα MAbs, the positive control MAb 2D2 and negative control MAb 16G5. After 30 minutes at 4 °C a constant amount of α-thrombin was added (0.75 µM final concentration). After 60 minutes incubation at 4 °C, wells were washed and residual bound α-thrombin was detected with chromogenic substrate S-2238.

4.2.4 Thrombin generation tests under static and flow conditions

The inhibitory effects of the antiplatelet antibodies on thrombin generation were studied under static and flow conditions using low TF as a stimulus as described in Section 2.8.

4.2.5 Expression of negatively charged phospholipids

The 'flip-flop' of the platelet membrane leading to the exposure of negatively charged phospholipids was studied using fluorescein isothiocyanate conjugated (FITC) annexin-5A. Annexin-5A binds to phosphatidylserine (PS), one of the negatively charged phospholipids exposed during the 'flip-flop' (Reutelingsperger, 2001).

4.2.5.1 Annexin-5A binding

Reaction mixtures were prepared as described for extrinsic thrombin generation (see Section 2.8.1.2). Thrombin generation was initiated by the addition of either low TF

(see Section 2.3) in 100 mM $CaCl_2$ or $CaCl_2$ alone for negative controls. Thrombin generation experiments had shown that maximum thrombin generation occurred after 12 minutes. At this time, 20 µl subsamples were taken into 180 µl HEPES-buffered saline (HBS; 0.01 M HEPES, 0.14 M NaCl, 0.025 M $CaCl_2$, pH 7.4) containing 20 µg/ml recombinant hirudin (Novartis, Basel, Switzerland), an approximate tenfold molar excess. Five µl samples of this mixture were mixed with 495 µl of HBS containing 1 µg of FITC-conjugated annexin-5A (Tau Technologies, Kattendijke, The Netherlands). The samples were incubated at room temperature for 10 minutes before analysis in a flow cytometer.

4.2.5.2 Flow cytometry

Binding of FITC-annexin-5A to the platelets was measured using a FACScan flow cytometer (Becton Dickinson, San Jose, USA). Platelets were distinguished from other blood cells and debris based on their forward and 90° light scatter profile (dot plot). A region was drawn around the platelet population and 10 000 events were analysed with the gain setting in the logarithmic mode. The threshold above which the platelets were considered positive was determined by setting a cursor on the histogram so that < 3% of the unstimulated platelets incubated with TBS and Ca^{2+} alone showed fluorescence above this level.

For each antibody treatment, the difference in the percentage of positive platelets between those stimulated with both TF and Ca^{2+} and those incubated with Ca^{2+} in the absence of TF was calculated (to correct for background levels caused by activation of platelets during handling). This value was then divided by that obtained from the

control (TBS treated) cells to obtain the percentage of control levels of annexin-5A binding.

4.2.6 Measurement of soluble fibrin in defibrinated plasma

The presence of soluble fibrin in defibrinated plasma was measured by ELISA (Edgell & Gaffney, 1996). A monoclonal capture antibody 5F3 (NIBSC in-house reagent), against fibrin and its fragments, was coated onto Maxisorp 96 well plates (Nunc Nalgene, from Invitrogen, Paisley, UK). Dilutions of the defibrinated plasma were prepared in citrate saline (150 mM NaCl, 109 mM trisodium citrate, BDH, Poole, UK) containing 1 mg/ml of the peptide Glycine-Proline-Arginine-Proline (Pentapharm, Basel, Switzerland) to inhibit cross-linking of fibrin. The sample dilutions were incubated in the coated wells before washing and detection with biotinylated MAb A11, against the intact carboxy terminal of the Aα chain of fibrin(ogen). The biotin was detected with streptavidin-HRP (Dako, Glostrup, Denmark), and o-toluidine (Sigma, Poole, UK) used as the substrate. Different defibrination methods were compared, including ancrod, (a venom protein from the snake *Agkistrodron rhodostroma* that cleaves fibrinopeptide A and slowly activates FXIII; Gaffney & Brasher, 1974), batroxobin (a venom protein from the snake *Bothrops atrox moojeni* that also cleaves fibrinopeptide A from fibrinogen, but does not activate FXIII (Gaffney & Edgell, 1998), immunoprecipitation and heat defibrination.

4.2.7 Immunofluorescent staining of platelets and HUVEC

In order to visualise the binding of RFGP56 and RFGP37 to platelets, and to investigate potential cross-reactivity of the anti-platelet antibodies with HUVEC, immunofluoresence was performed as follows.

Platelet plasma was prepared as detailed in Section 2.7 and incubated for 30 minutes at 37 °C with 20 μg/ml of RFGP56, Serotec RFGP37 or rabbit anti-vWF polyclonal antibody (Dako, Glostrup, Denmark). A drop of the cell suspension was then applied to a glass coverslip and washed, fixed and stained as described in Section 2.11. HUVEC were cultured on Thermanox coverslips as described in Section 2.9, washed, fixed and incubated with antibodies, as before. The secondary antibodies were FITC rabbit anti-mouse or FITC swine anti-rabbit IgG (both from Dako, Glostrup, Denmark) and stains for actin and for nuclear material were also included, as detailed in Section 2.11.

4.3 Results

4.3.1 Platelet aggregation

The anti-GP IIb/IIIa antibody RFGP56 inhibited platelet aggregation with a final thrombin concentration of 0.5 IU/ml in a dose-dependent manner. Aggregation was reduced to 68.2 ± 3.2, 24.8 ± 9.5 and 19.0 ± 6.2 % with 5, 10 and 20 μg/ml RFGP56 respectively (all $p < 0.01$; see Figure 4.1). Similar inhibition was seen with c7E3 at 20 μg/ml, reducing platelet aggregation to 24.7 ± 6.2 % ($p < 0.01$). The anti-GP Ibα antibody RFGP37 did not inhibit platelet aggregation at 20 μg/ml (Cymbus 113.0 ± 29.6 %, ns; Serotec 136.0 ± 77.2, % not shown) or at concentrations as high as 120 μg/ml (Cymbus 137.7 ± 28.8 %, not shown).

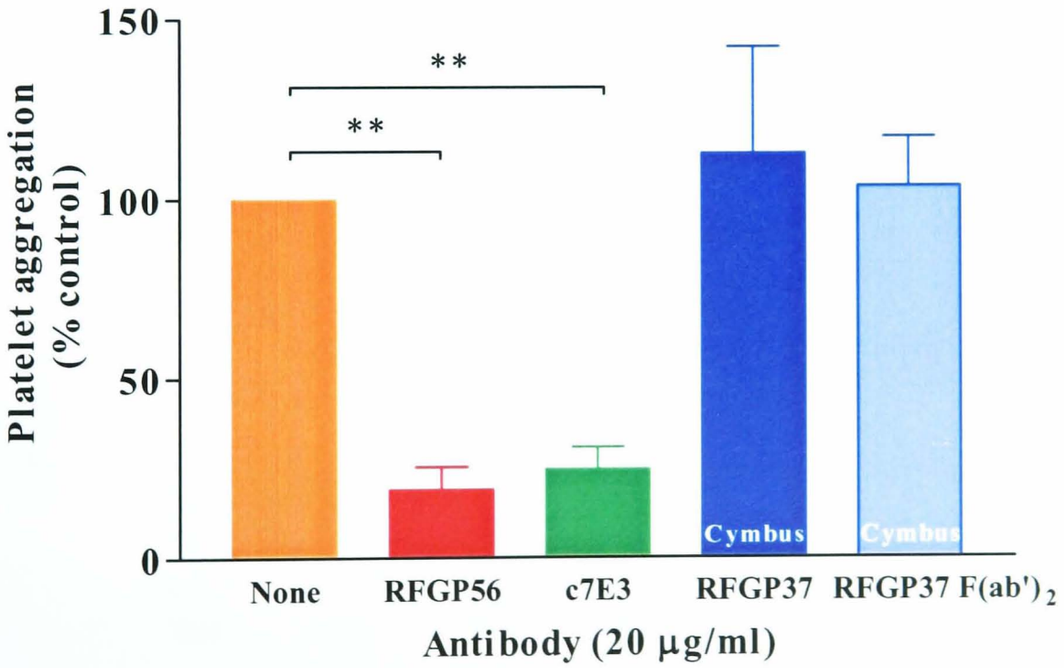


Figure 4.1 *Platelet aggregation in the presence of anti-platelet antibodies.*

*Washed platelets were stimulated with 0.5 IU/ml (final concentration) human α-thrombin following a 5 minute incubation with the antibody under test. Results were calculated as a percentage of the aggregation seen in the absence of antibody 5 minutes after the addition of thrombin. Mean ± sd, $n \geq 3$, ** $p < 0.01$.*

$F(ab')_2$ fragments of Cymbus RFGP37 also failed to inhibit thrombin-induced aggregation (103.8 ± 13.7 % at 20 μg/ml). Control antibody RegA had no effect on aggregation at 20 μg/ml (93.7 ± 5.8 %).

As shown in Figure 4.2, when concentrations of thrombin lower than 0.5 IU/ml were used, Cymbus RFGP37 became able to inhibit platelet aggregation. Increased inhibition was seen with 20 μg/ml of Cymbus RFGP37 in response to progressively lower concentrations of thrombin. Serotec RFGP37 did not produce this inhibition. This effect was not seen with 20 μg/ml of RFGP56, which gave maximal inhibition of aggregation at all concentrations of thrombin.

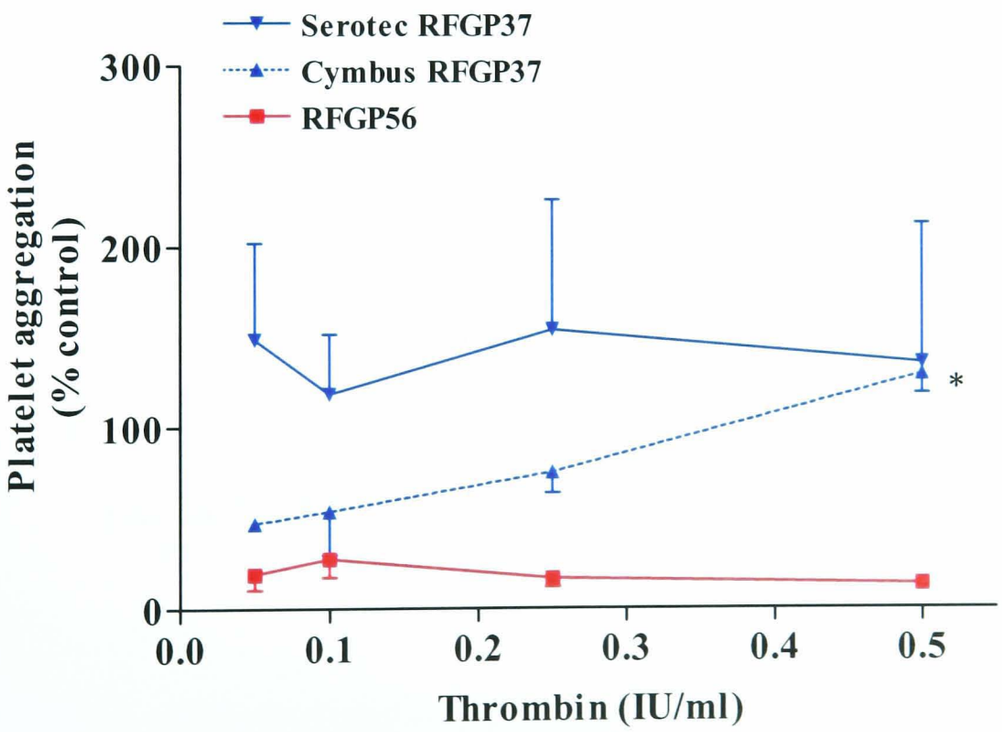


Figure 4.2 *Platelet aggregation with dilutions of thrombin.*

*Washed platelets were incubated with 20 μg/ml RFGP56 or RFGP37 and then stimulated with thrombin at 0.05 – 0.5 IU/ml final concentration. Results are expressed as percentage aggregation compared to control samples (without antibodies). Mean ± sd; n ≥ 3; *r^2 = 0.99.*

4.3.2 ELISA to evaluate the specificity and inhibition of thrombin binding of anti-GP Ibα antibodies

Figure 4.3 shows that different batches of RFGP37 have differing abilities to bind to the recombinant fragment of GP Ibα consisting of residues 1 – 289 (GP $Ib\alpha_{1-189}$). The batch that shows the best inhibition (ST0802) was purified using Protein G from cell culture supernatant frozen in 1994. All other batches were produced more recently by the biotechnology firms Cymbus and Serotec, using freshly grown hybridoma cells. The original batch of Cymbus RFGP37 used in platelet aggregation, flow cytometry and thrombin generation experiments (batch 297J) was not available for testing. The Cymbus batches shown in Figure 4.3 were the replacement batches supplied when stocks of 297J were exhausted.

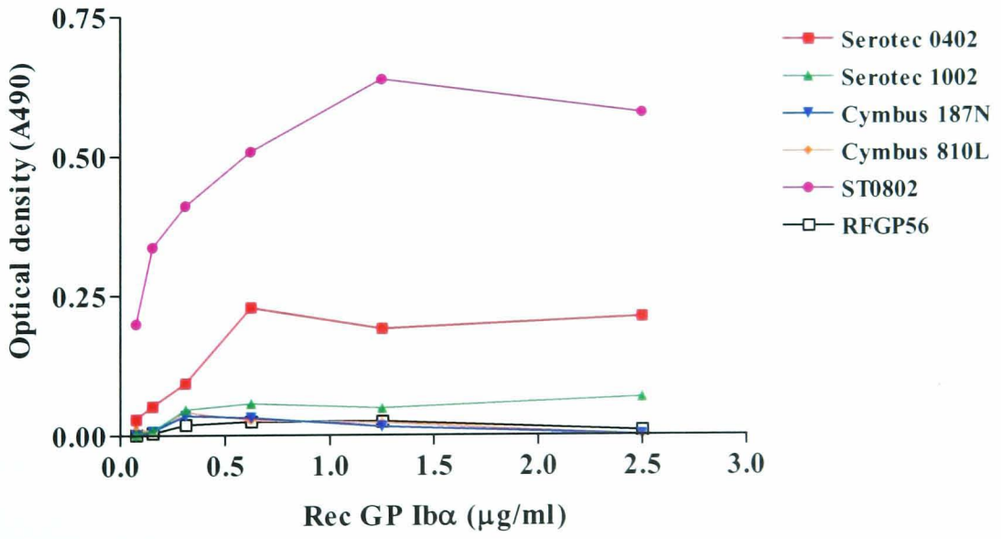


Figure 4.3 *Binding of anti-platelet antibodies to recombinant GP $Ib\alpha_{1-289}$.*

Five µg/ml of each of five batches of RFGP37 and a single batch of RFGP56 were coated onto a microtitre plate and incubated with varied amounts of recombinant GP $Ib\alpha_{1-289}$ for 2 hours at 37 °C. Bound GP$Ib\alpha_{1-289}$ was detected with 1 µg/ml biotinylated anti-GP Ibα MAbs 6B4 and 12G1 for 90 minutes at room temperature before incubation with strepdavidin conjugated horseradish peroxidase for 45 minutes at room temperature. Visualisation was with ortho-phenyldiamine after the reaction was stopped with 4 M H_2SO_4. Data are from a single experiment, performed by Mr Hans Ulrichts of The University of Leuven, Kortrijk, Belgium.

Figure 4.4 shows that different batches of RFGP37 have a variety of inhibitory activities against the binding of thrombin to glycocalicin or the recombinant fragment of GP Ib$\alpha_{1\text{-}289}$. The data are from a single assay, making statistical analysis impossible, but it would be impossible to claim that any specific inhibition of thrombin binding was evident.

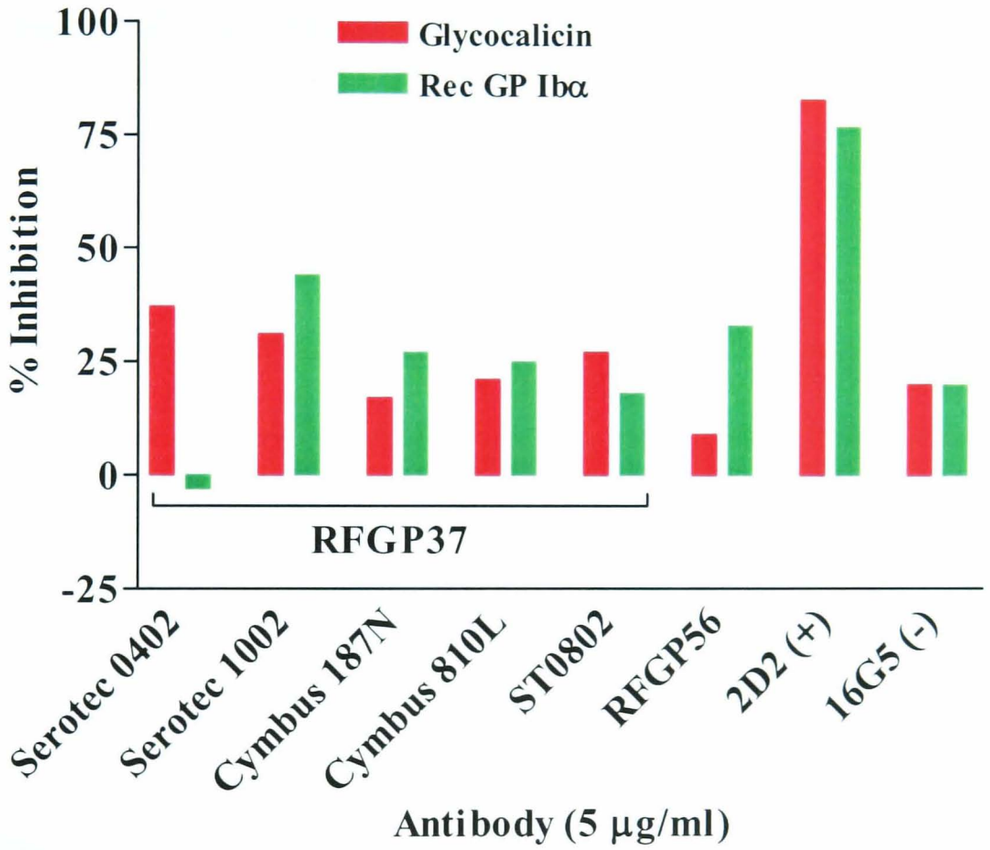


Figure 4.4 *The ability of anti-platelet antibodies to inhibit the binding of thrombin to glycocalicin or recombinant GP Ib$\alpha_{1\text{-}289}$.*

A microtitre plate was coated with 5 µg/ml of anti GPIbα MAb 12G1 followed by 1 µg/ml recominant GP Ibα. A dilution series of five batches of RFGP37, one batch of RFGP56 and positive (2D2) and negative (16G5) controls were added to the wells and incubated for 30 minutes at 4 °C before the addition of 0.75 µM α-thrombin. After 60 minutes incubation at 4 °C the bound thrombin was detected with chromogenic substrate S2238. Data are from a single experiment, performed by Mr Hans Ulrichts, University of Leuven, Kortrijk, Belgium.

4.3.3 Thrombin generation under static conditions following intrinsic stimulation

Thrombin generation was performed in polystyrene tubes, using kaolin as the stimulus (as detailed in Section 2.8.1.1), and the clotting system for thrombin determination (see Section 2.8.4.1). Thrombin generation curves from sample experiments are shown in Figure 4.5a to illustrate the effect of the antibodies on thrombin generation. No thrombin was detectable in the absence of platelets, indicating that this was a platelet-dependent system.

From the platelet aggregation data in Section 4.3.1 and from previously published data for c7E3 (Reverter *et al*, 1996), 20 μg/ml was judged to be a saturating concentration of antibody and selected for study in the thrombin generation test. Figure 4.5b shows that at 20 μg/ml antibodies against both GP IIb/IIIa and GP Ibα inhibited total thrombin generation in this system, measured by AUC. RFGP56 reduced thrombin generation to 25.1 ± 2.9 % ($p < 0.01$) of control levels but c7E3 was less effective, reducing thrombin generation to 50.4 ± 22.4 % ($p < 0.01$). Cymbus RFGP37 reduced thrombin generation to 69.1 ± 3.9 % of control levels ($p < 0.05$). No further inhibition was seen in a single experiment using 40 μg/ml of either RFGP56 or RFGP37. Figure 4.5c shows that similar effects were seen when peak thrombin generation was analysed. No significant differences were seen in the time of the thrombin peak in the presence of any antibody when compared with the control (Figure 4.5d).

Fig 4.5a

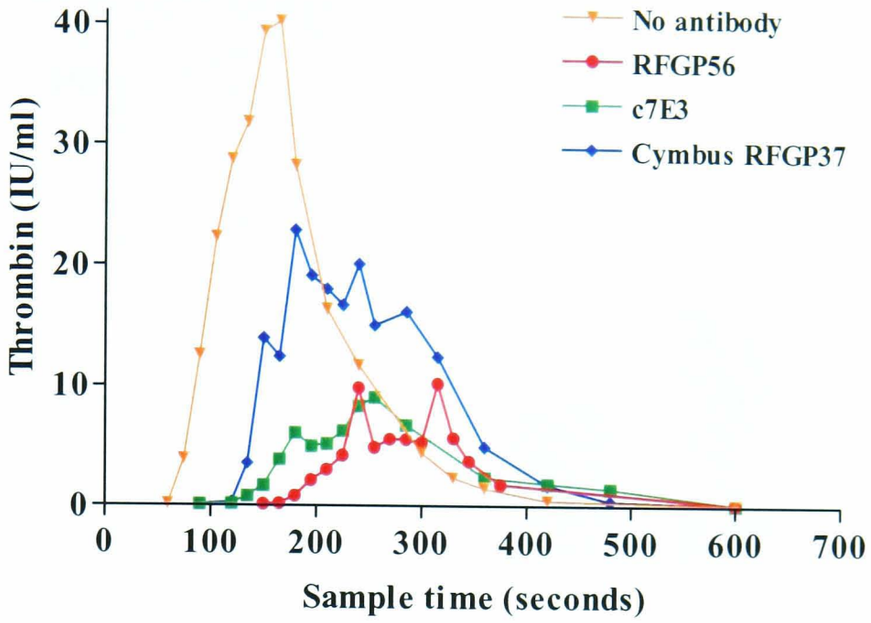


Fig 4.5b

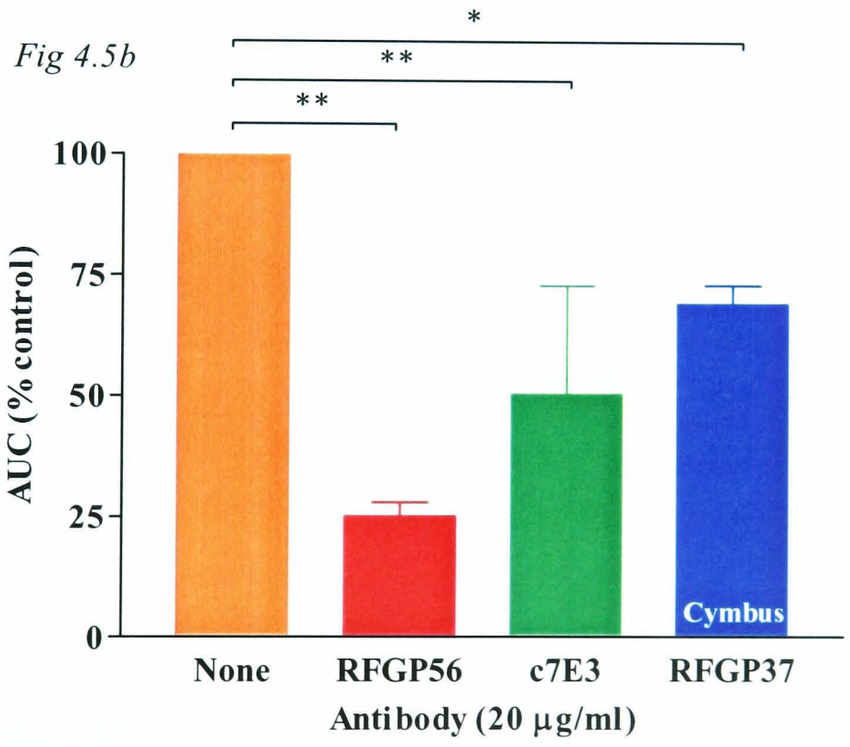


Figure 4.5 *Inhibition of thrombin generation by anti-platelet antibodies following intrinsic stimulation under static conditions.*

800 µl of defibrinated plasma containing 300 x 10^6 platelets was incubated for 20 minutes at 37 °C with 25 µl of TBS containing the antibody under test and 100 µl of 100 mg/ml kaolin suspension in TBS. Thrombin generation was initiated with 75 µl of 200 mM $CaCl_2$ and timed subsamples were taken into fibrinogen for determination of thrombin concentration.

*a) Representative thrombin generation curves from single experiments and the effect of antibodies on b) AUC, c) peak thrombin and d) time of peak thrombin. *$p < 0.05$, **$p < 0.01$.*

Fig 4.5c

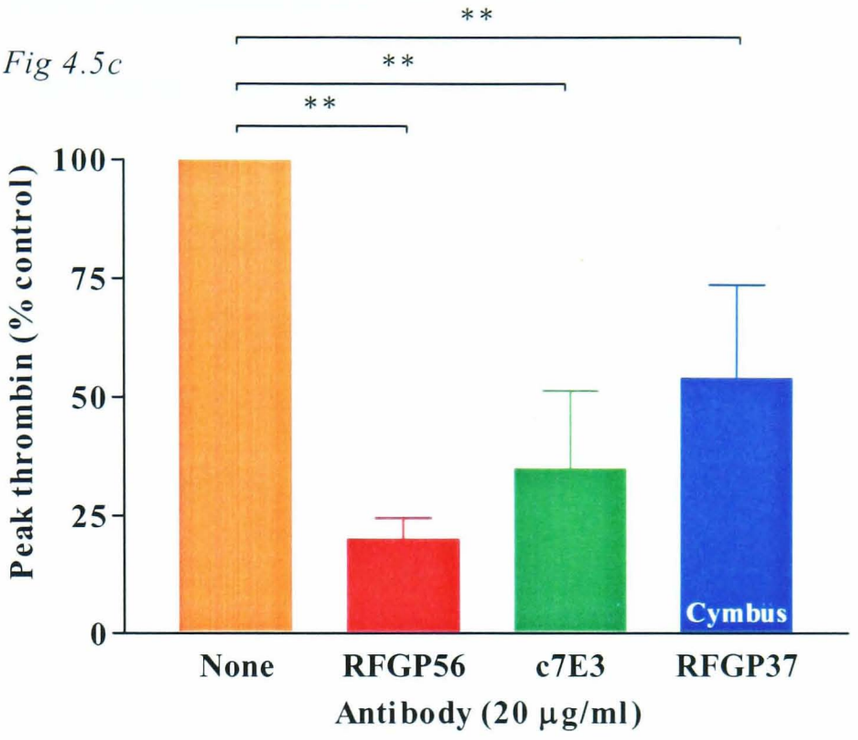


Fig 4.5d

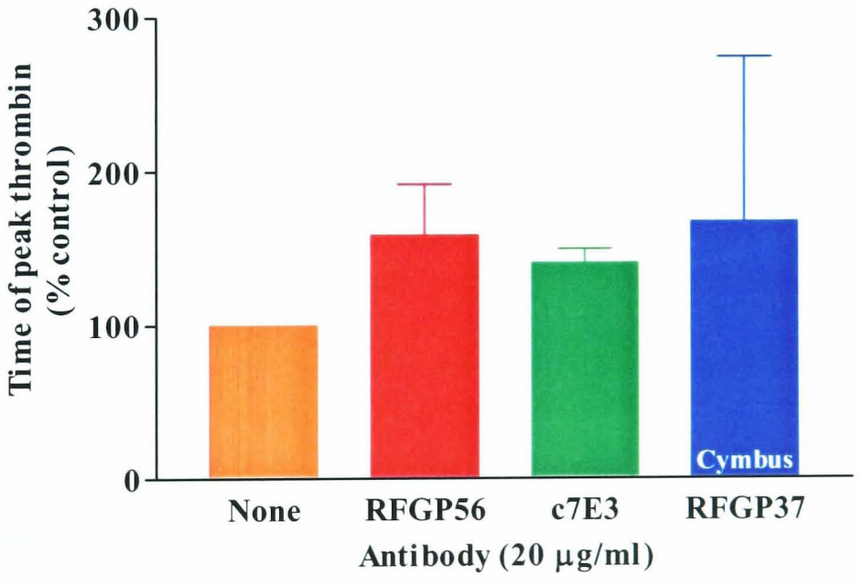


Figure 4.5 *Inhibition of thrombin generation by anti-platelet antibodies following intrinsic stimulation under static conditions.*

800 µl of defibrinated plasma containing 300 x 10^6 platelets was incubated for 20 minutes at 37 °C with 25 µl of TBS containing the antibody under test and 100 µl of 100 mg/ml kaolin suspension in TBS. Thrombin generation was initiated with 75 µl of 200 mM $CaCl_2$ and timed subsamples were taken into fibrinogen for determination of thrombin concentration.

*a) Representative thrombin generation curves from single experiments and the effect of antibodies on b) AUC, c) peak thrombin and d) time of peak thrombin. *$p < 0.05$, **$p < 0.01$.*

4.3.4 Thrombin generation under static conditions following high TF stimulation

Thrombin generation was performed in polystyrene tubes as detailed in Section 2.8.1.2, using high TF as the stimulus (see Section 2.3) and the clotting system for thrombin determination, as detailed in Section 2.8.4.1. Thrombin generation curves from sample experiments are shown in Figure 4.6a to illustrate the effect of the antibodies on thrombin generation and shows that all of the antibodies tested had reduced inhibitory effects compared to those in the intrinsic system described in Section 4.3.3. At 20 μg/ml, RFGP56, c7E3 and Cymbus RFGP37 caused reductions in thrombin generation measured by AUC to 76.6 ± 13.6 ($p < 0.05$), 86.6 ± 2.0 (ns) and 83.4 ± 6.0 % (ns) of control levels respectively. In the absence of platelets, the addition of high TF resulted in 63.2 ± 17.2 % of control levels of thrombin being generated, suggesting that only approximately 40 % of thrombin generation in this system is platelet dependent (Figure 4.6b). Analysis of the peak concentration of thrombin gave similar results to the AUC data, with 20 μg/ml of RFGP56, c7E3 and Cymbus RFGP37 reducing the peak to 64.4 ± 12.0 ($p < 0.05$), 80.6 ± 21.8 (ns) and 73.3 ± 9.4 % (ns) of control levels respectively. In the absence of platelets, 52.2 ± 5.6 % of the peak thrombin concentration in control experiments was generated, confirming the significance of platelet-independent thrombin generation in this system (Figure 4.6c). The time of the peak concentration of thrombin was not significantly affected by the presence of antiplatelet antibodies but tended to be later at 138.2 ± 44.4, 151.9 ± 46.8 and 131.1 ± 19.9 with RFGP56, c7E3 and Cymbus RFGP37 respectively. In the absence of platelets the peak was seen at 151.9 ± 45.6 % of the time in the presence of platelets and, although this was not significant, it may suggest that platelets accelerate the generation of thrombin in this system (Figure 4.6d).

Fig 4.6a

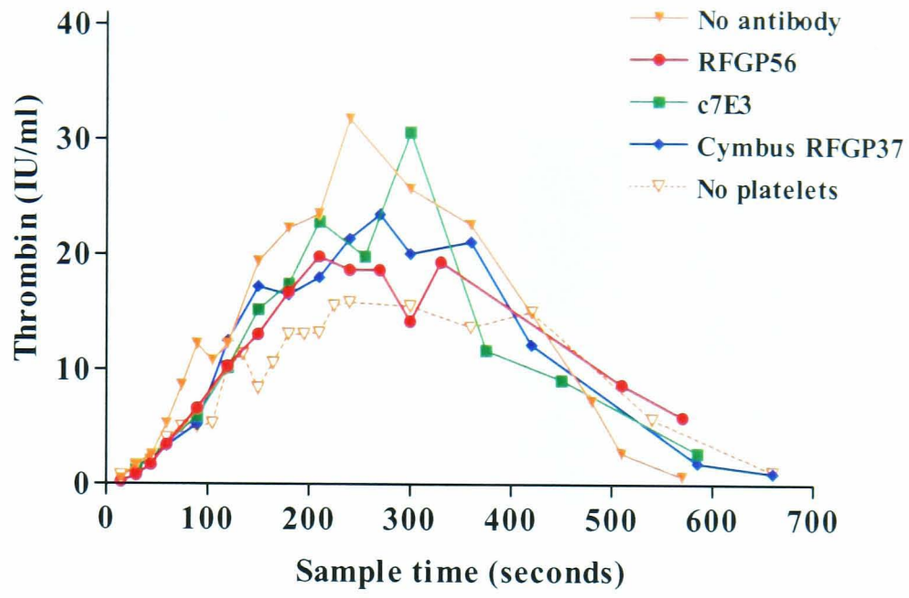


Fig 4.6b

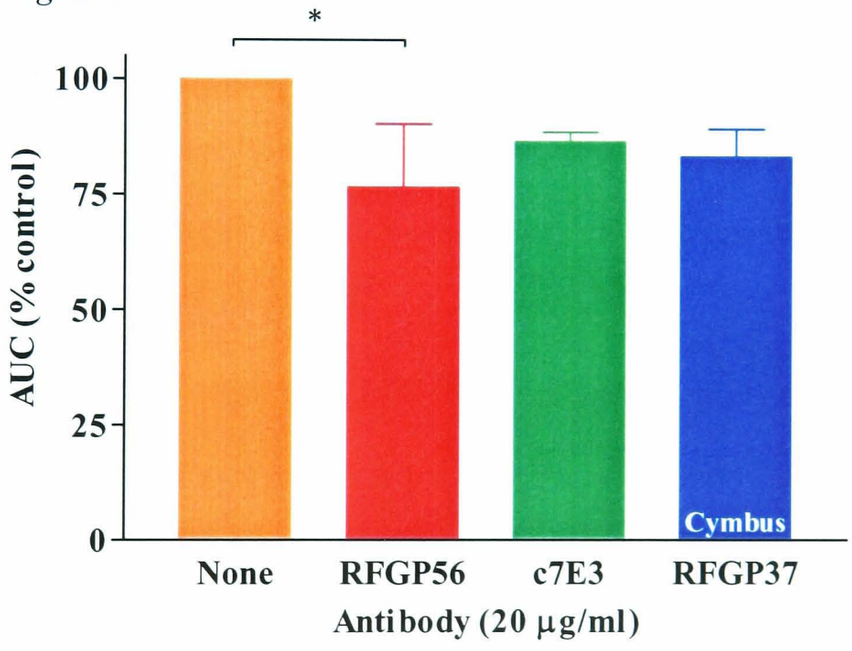


Figure 4.6 *Inhibition of thrombin generation by anti-platelet antibodies following extrinsic stimulation with high TF under static conditions.*

800 µl of defibrinated plasma containing 300 x 10^6 platelets was incubated for 30 minutes at 37 °C with 25 µl of TBS containing the antibody under test. Thrombin generation was initiated with 175 µl of 100 mM $CaCl_2$ containing recombinant tissue factor to give a final concentration of 630 pM. Timed subsamples were taken into fibrinogen for determination of thrombin concentration.

*a) Representative thrombin generation curves from single experiments and the effect of antibodies on b) AUC, c) peak thrombin and d) time of peak thrombin. *$p < 0.05$.*

Fig 4.6c

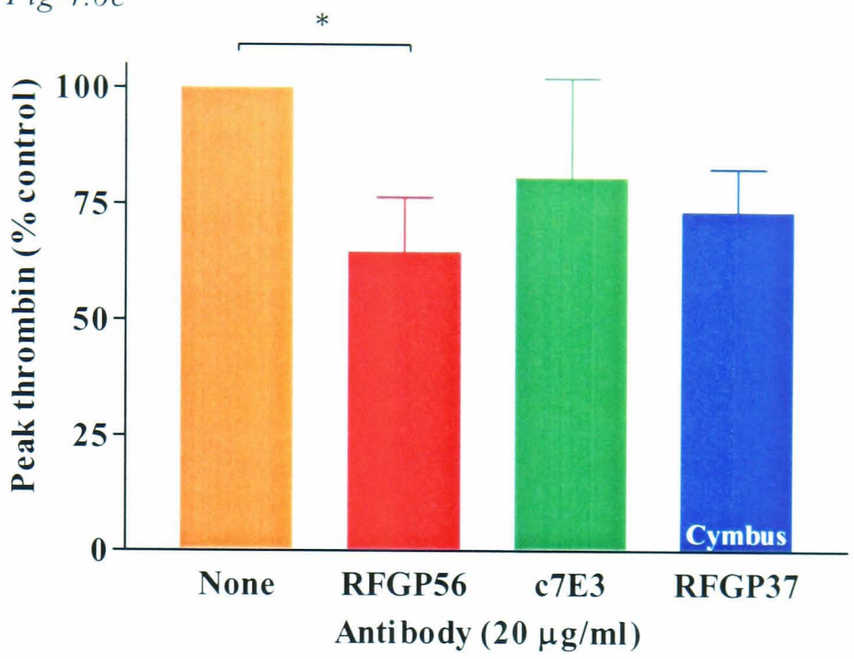


Fig 4.6d

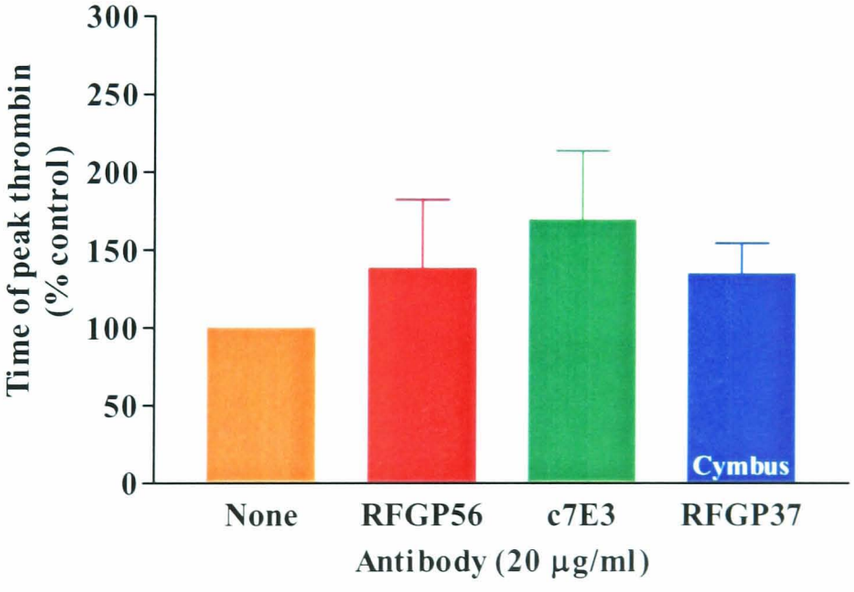


Figure 4.6 *Inhibition of thrombin generation by anti-platelet antibodies following extrinsic stimulation with high TF under static conditions.*

800 µl of defibrinated plasma containing 300 x 10^6 platelets was incubated for 30 minutes at 37 °C with 25 µl of TBS containing the antibody under test. Thrombin generation was initiated with 175 µl of 100 mM $CaCl_2$ containing recombinant tissue factor to give a final concentration of 630 pM. Timed subsamples were taken into fibrinogen for determination of thrombin concentration.

*a) Representative thrombin generation curves from single experiments and the effect of antibodies on b) AUC, c) peak thrombin and d) time of peak thrombin. *$p < 0.05$.*

4.3.5 Thrombin generation under static conditions following low TF stimulation

Thrombin generation was performed in polystyrene tubes as detailed in Section 2.8.1.2, using low TF as the stimulus (see Section 2.3) and the clotting system for thrombin determination, as detailed in Section 2.8.4.1. Anti-GP IIb/IIIa antibodies had similar inhibitory effects in this system compared to the intrinsic system (see Table 4.1). Thrombin generation curves from sample experiments are shown in Figure 4.7a to illustrate the effect of the antibodies on thrombin generation. At 20 μg/ml, RFGP56 and c7E3 reduced thrombin generation determined by AUC to 47.3 ± 5.9 % and 59.9 ± 21.2 % of the control level respectively ($p < 0.01$; Figure 4.7b). Five μg/ml Cymbus RFGP37 reduced AUC thrombin generation to 68.5 ± 14.3 % of the control level ($p < 0.01$), but there was no further increase at 20 μg/ml (81.0 ± 1.3 %; ns). A dose response was seen with RFGP56, but linear regression showed this to be not significant ($r^2 = 0.44$; $p = 0.052$). No significant dose response was seen with RFGP37. All antibodies were able to reduce the peak concentration of thrombin generated. Twenty μg/ml of RFGP56, c7E3 and RFGP37 reduced the peak to 23.8 ± 5.7, 29.1 ± 13.0 and 74.9 ± 3.6 % of control values (all $p < 0.01$; Figure 4.7c) and RFGP56 reduced the peak thrombin generation in a dose-dependent manner ($r^2 = 0.52$; $p < 0.05$, not shown). None of the antibodies was able to delay significantly the peak of the thrombin generation curve (Figure 4.7d).

Table 4.1 *Inhibition of thrombin generation by anti-platelet antibodies following different methods of stimulation.*

*Intrinsic stimulation was achieved by incubating 800 µl of defibrinated plasma containing 300 x 10^6 platelets for 20 minutes at 37 °C with 25 µl of TBS containing the antibody under test and 100 µl of 100 mg/ml kaolin suspension in TBS. Thrombin generation was initiated with 75 µl of 200 mM $CaCl_2$. Extrinsic stimulation was achieved by incubating 800 µl of defibrinated plasma containing 300 x 10^6 platelets for 30 minutes at 37 °C with 25 µl of TBS containing the antibody under test. Thrombin generation was initiated with 175 µl of 100 mM $CaCl_2$ containing recombinant tissue factor to give a final concentration of either 630 or 35 pM for 'high' or 'low' TF respectively. In all cases timed subsamples were taken into fibrinogen for determination of thrombin concentration. Data are AUC as % control; mean ± sd; n ≥ 3; *p < 0.05; **p < 0.01*

Stimulus	Antibody (20 µg/ml)		
	RFGP56	c7E3	RFGP37
Intrinsic	25.1 ± 2.9 **	50.4 ± 22.4 **	69.1 ± 3.9 *
High TF Extrinsic	74.6 ± 13.6 **	86.6 ± 2.0	83.4 ± 6.0
Low TF Extrinsic	47.3 ± 5.9 **	59.9 ± 21.2 **	81.0 ± 1.3

Fig 4.7a

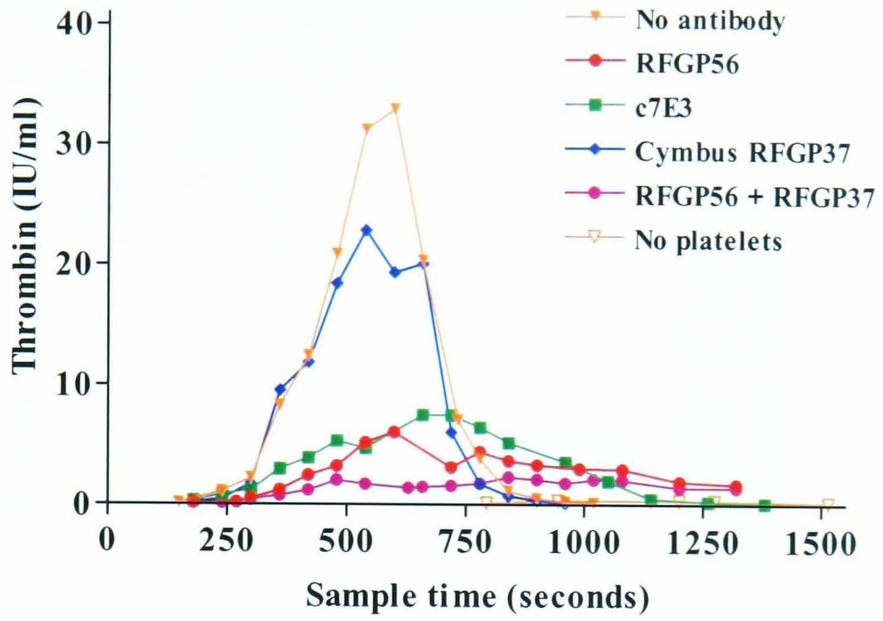


Fig 4.7b

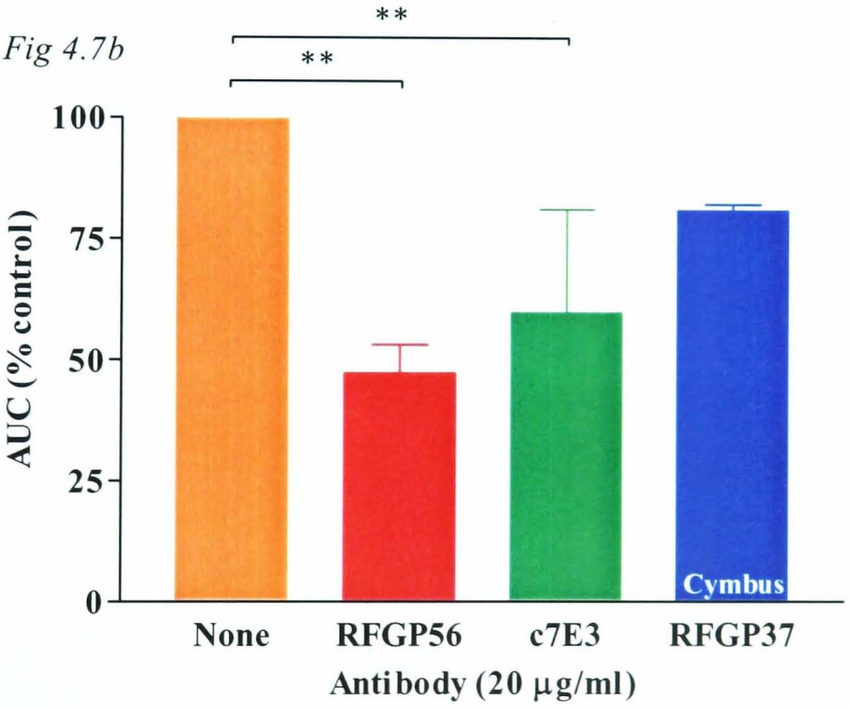


Figure 4.7 *Inhibition of thrombin generation by anti-platelet antibodies following extrinsic stimulation with low TF under static conditions.*

800 µl of defibrinated plasma containing 300 x 10^6 platelets was incubated for 30 minutes at 37 °C with 25 µl of TBS containing the antibody under test. Thrombin generation was initiated with 175 µl of 100 mM $CaCl_2$ containing recombinant tissue factor to give a final concentration of 35 pM. Timed subsamples were taken into fibrinogen for determination of thrombin concentration.

*a) Representative thrombin generation curves from single experiments and the effect of antibodies on b) AUC, c) peak thrombin and d) time of peak thrombin. **$p < 0.01$.*

Fig 4.7c

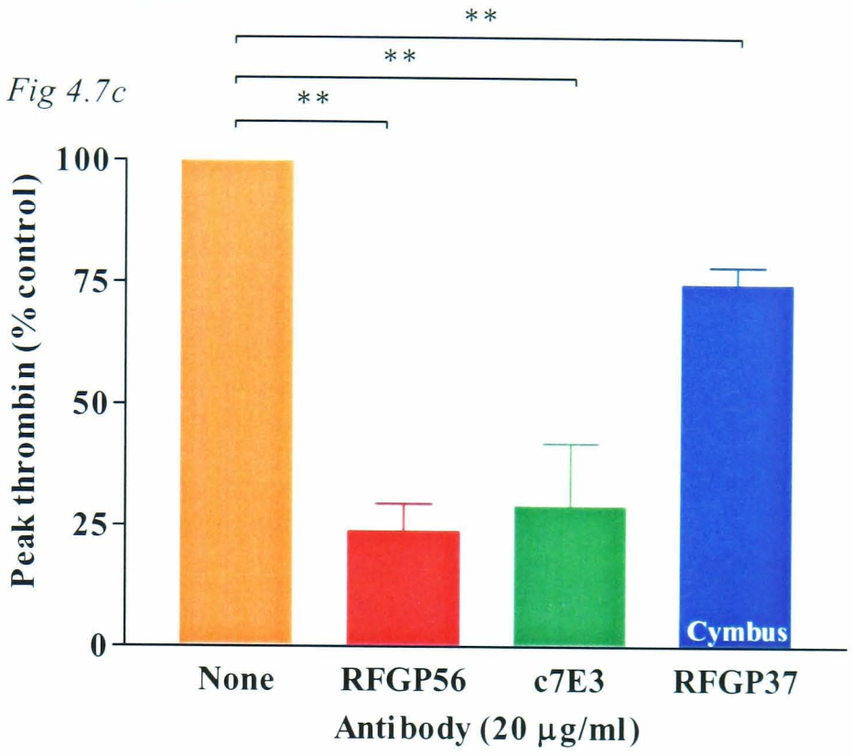


Fig 4.7d

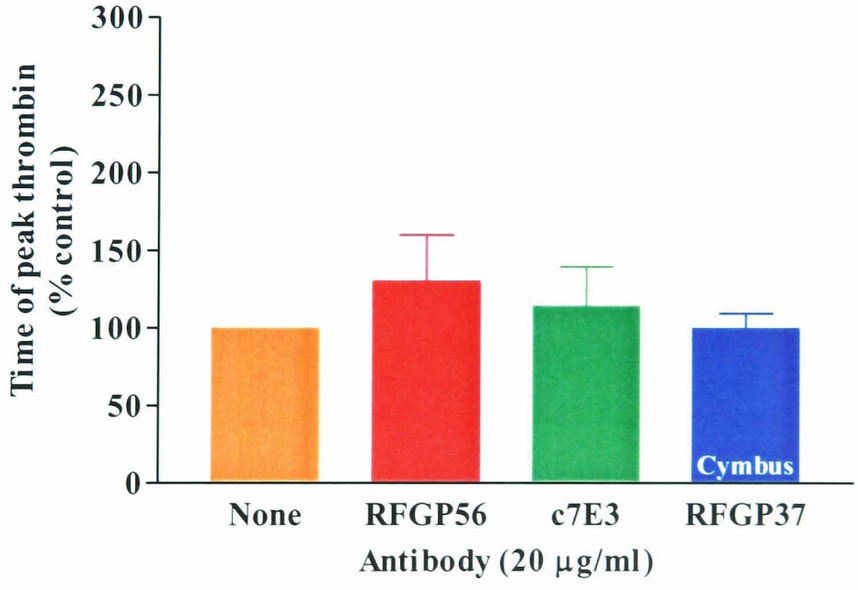


Figure 4.7 *Inhibition of thrombin generation by anti-platelet antibodies following extrinsic stimulation with low TF under static conditions.*

800 µl of defibrinated plasma containing 300 x 10^6 platelets was incubated for 30 minutes at 37 °C with 25 µl of TBS containing the antibody under test. Thrombin generation was initiated with 175 µl of 100 mM $CaCl_2$ containing recombinant tissue factor to give a final concentration of 35 pM. Timed subsamples were taken into fibrinogen for determination of thrombin concentration.

*a) Representative thrombin generation curves from single experiments and the effect of antibodies on b) AUC, c) peak thrombin and d) time of peak thrombin. **$p < 0.01$.*

In the absence of platelets, none of the antibodies showed significant inhibition of thrombin generation following stimulation under static conditions with low TF (see Figure 4.8).

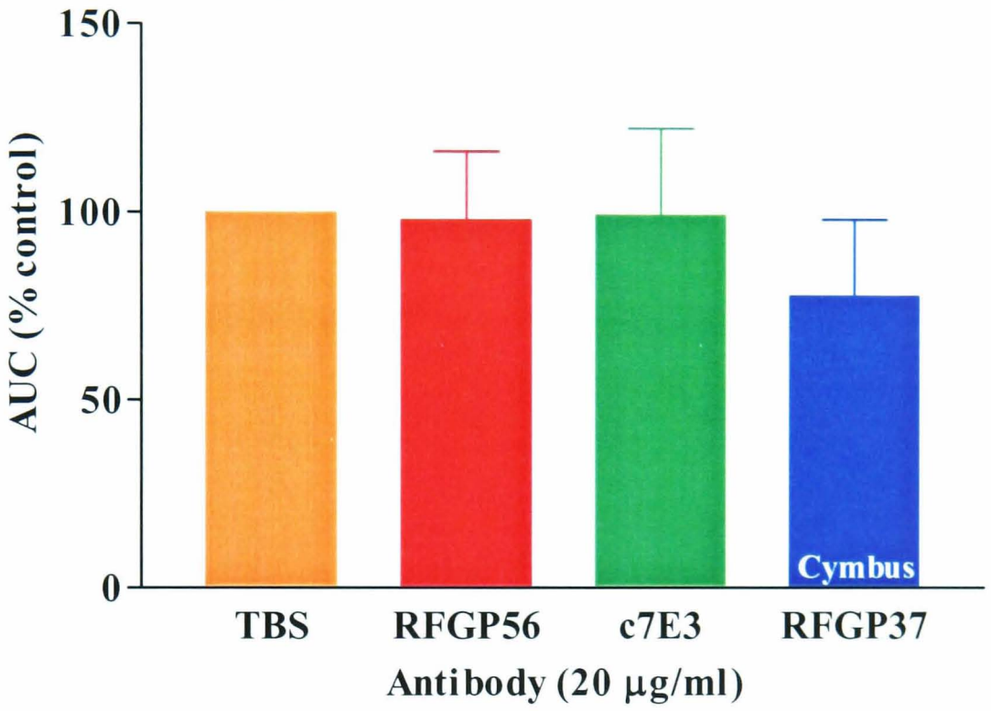


Figure 4.8 *Effect of anti-platelet antibodies on thrombin generation following low TF stimulation in platelet poor plasma.*

800 µl of defibrinated plasma without platelets was incubated for 30 minutes at 37 °C with 25 µl of TBS containing the antibody under test. Thrombin generation was initiated with 175 µl of 100 mM $CaCl_2$ containing recombinant tissue factor to give a final concentration of 35 pM. Timed subsamples were taken into fibrinogen for determination of thrombin concentration. Mean ± sd; n = 3.

It has previously been reported that concentrations of c7E3 above 10 µg/ml (200 nM of monovalent Fab) inhibited prothrombinase activity when phospholipid vesicles were used in place of platelets (Pedicord *et al*, 1998). Experiments were therefore performed using a phospholipid preparation (NIBSC 91/542, diluted 1/125 in TBS) in place of platelets. No significant inhibition of thrombin generation was seen with 20 µg/ml (133 nM of bivalent IgG) of RFGP56 (96.8 ± 18.3 %), Cymbus RFGP37 (76.0 ± 20.5 %) or c7E3 (97.4 ± 23.1 %); data not shown.

In combination, RFGP56 and Cymbus RFGP37 had an additive effect, with 20 μg/ml of each antibody reducing thrombin generation (AUC) to 28.1 ± 12.6 % of the control level ($p < 0.01$). This effect was dose dependent ($r^2 = 0.49$; $p < 0.01$; see Figure 4.9a) as 10 and 5 μg/ml of both antibodies together reduced thrombin generation (AUC) to 46.6 ± 19.7 % and 77.6 ± 16.4 % respectively. A similar dose-dependent reduction of peak thrombin generation was seen with both antibodies together ($r^2 = 0.7$; $p < 0.001$; see Figure 4.9b), but no effect was seen on the time of the peak of the thrombin generation curve (Figure 4.9c).

Fig 4.9a

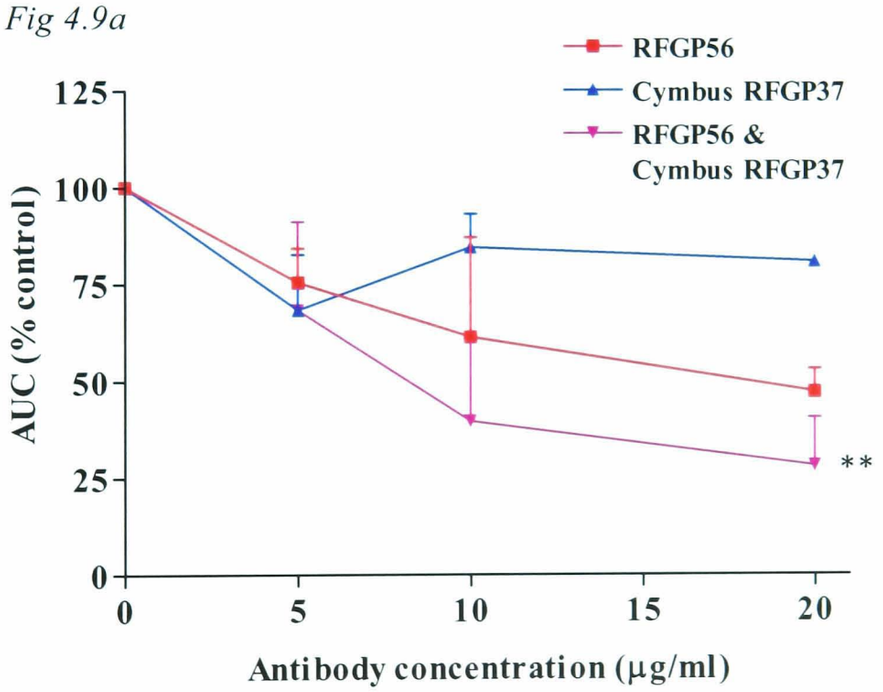


Figure 4.9 *Dose response curves showing inhibition of thrombin generation by RFGP37 and RFGP56 following extrinsic stimulation with low TF under static conditions.*

800 μl of defibrinated plasma containing 300 x 10^6 platelets was incubated for 30 minutes at 37 °C with 25 μl of TBS containing the antibody under test. Thrombin generation was initiated with 175 μl of 100 mM $CaCl_2$ containing recombinant tissue factor to give a final concentration of 35 pM. Timed subsamples were taken into fibrinogen for determination of thrombin concentration.

a) AUC b) peak thrombin and c) time of peak thrombin.

*Mean ± sd; *p < 0.05, **p < 0.01, ***p < 0.001.*

Fig 4.9b

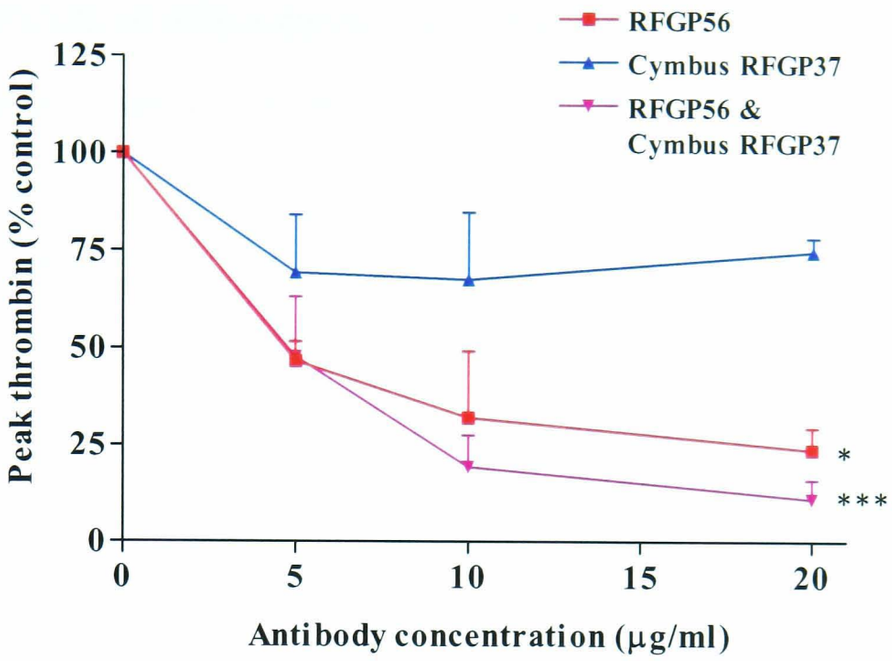


Fig 4.9c

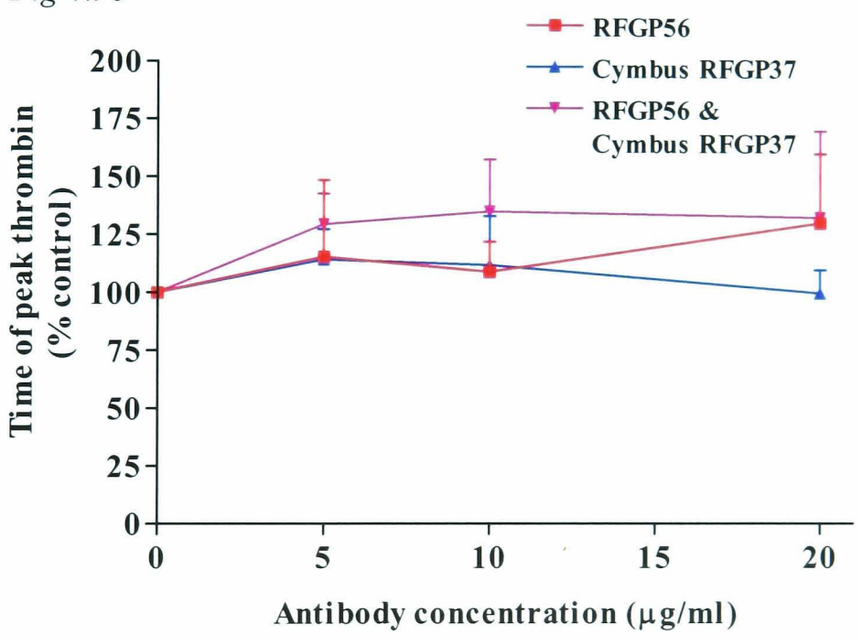


Figure 4.9 *Dose response curves showing inhibition of thrombin generation by RFGP37 and RFGP56 following extrinsic stimulation with low TF under static conditions.*

800 µl of defibrinated plasma containing 300 x 10^6 platelets was incubated for 30 minutes at 37 °C with 25 µl of TBS containing the antibody under test. Thrombin generation was initiated with 175 µl of 100 mM $CaCl_2$ containing recombinant tissue factor to give a final concentration of 35 pM. Timed subsamples were taken into fibrinogen for determination of thrombin concentration.

a) AUC b) peak thrombin and c) time of peak thrombin.

*Mean ± sd; *p < 0.05, **p < 0.01, ***p < 0.001.*

4.3.6 Thrombin generation under static or flow conditions in the presence of HUVEC or ECM following low TF extrinsic stimulation

Thrombin generation was performed as detailed in Section 2.8.1.2, using low TF as the stimulus (see Section 2.3), in the presence of HUVEC or ECM under static or flow conditions, as detailed in Sections 2.8.2 and 2.8.3 respectively. The chromogenic system detailed in Section 2.8.4.2 was used for thrombin determination.

Following on from the static studies that established the low TF extrinsic stimulation as a platelet-dependent thrombin generation system, the antiplatelet antibodies were studied in parallel plate flow chambers (described in Section 3.2.4.2) to examine their inhibitory effects under flow conditions. Early studies were performed using the Type 1 flow chamber at a relatively low shear rate of 18 s^{-1} before the Type 2 chambers became available for later studies at shear rates up to 600 s^{-1}. The floor of the flow chambers comprised a tissue culture slide or coverslip upon which HUVEC had been cultured (and sometimes removed to expose ECM), and in order to make valid comparisons with results obtained under flow conditions, static experiments were repeated using culture wells containing HUVEC or ECM.

4.3.6.1 RFGP56

When thrombin generation was measured under static conditions in the presence or absence of HUVEC, there was no significant difference in the inhibitory effect of RFGP56. Twenty μg/ml RFGP56 inhibited AUC to 54.2 ± 10.2 % of control values in the presence of HUVEC, compared to 47.3 ± 5.9 % in the absence of HUVEC in polystyrene test tubes (as reported in Section 4.3.5, using the clotting method of thrombin determination). The introduction of shear stress led to reduced inhibition in

the presence of HUVEC by 20 μg/ml RFGP56, to 88.6 ± 22.4, 81.8 ± 5.7 and 65.2 ± 19.2 % of control AUC at 18, 178 and 600 s^{-1} respectively (Figure 4.10b). Increasing the concentration of RFGP56 up to 80 μg/ml did not have any further inhibitory effect, but over the full range of concentrations up to 80 μg/ml, ANOVA revealed that RFGP56 was a significantly better inhibitor under static (0 s^{-1}) and arterial conditions (600 s^{-1}) when compared with venous conditions (18 and 178 s^{-1}). This was the case when measuring AUC and peak thrombin concentration (Figure 4.10c), but no significant differences were noted for the time of the peak (Figure 4.10d).

In the presence of ECM, thrombin generation under static conditions was reduced to 70.0 ± 18.6 % by 20 μg/ml RFGP56 (see Figure 4.11). The introduction of shear stress in the presence of ECM abolished the significance of this inhibitory effect, with AUC of 110.7 ± 19.7, 79.6 ± 22.8 and 72.4 ± 20.6 at 18, 178 and 600 s^{-1} respectively (Figure 4.11a). Increasing the concentration of RFGP56 up to 80 μg/ml did not have any further inhibitory effect, but ANOVA revealed that RFGP56 was more effective at reducing the peak thrombin concentration under static (0 s^{-1}) and arterial conditions (600 s^{-1}) when compared with venous conditions (18 and 178 s^{-1}; Figure 4.11c). No significant differences were noted for AUC (Figure 4.11b) or time of peak thrombin (Figure 4.11d).

Fig 4.10a HUVEC + RFGP56

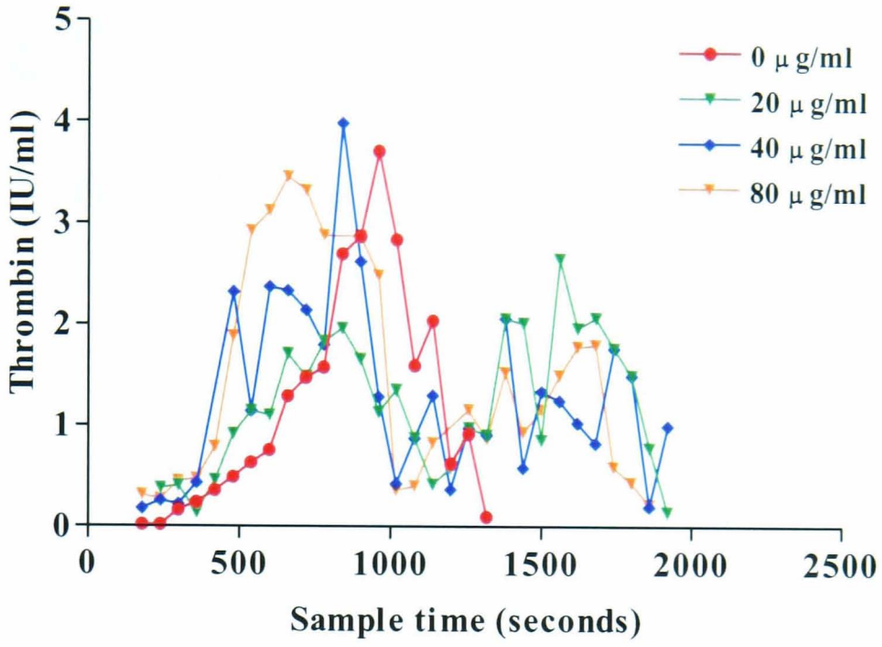


Fig 4.10b HUVEC

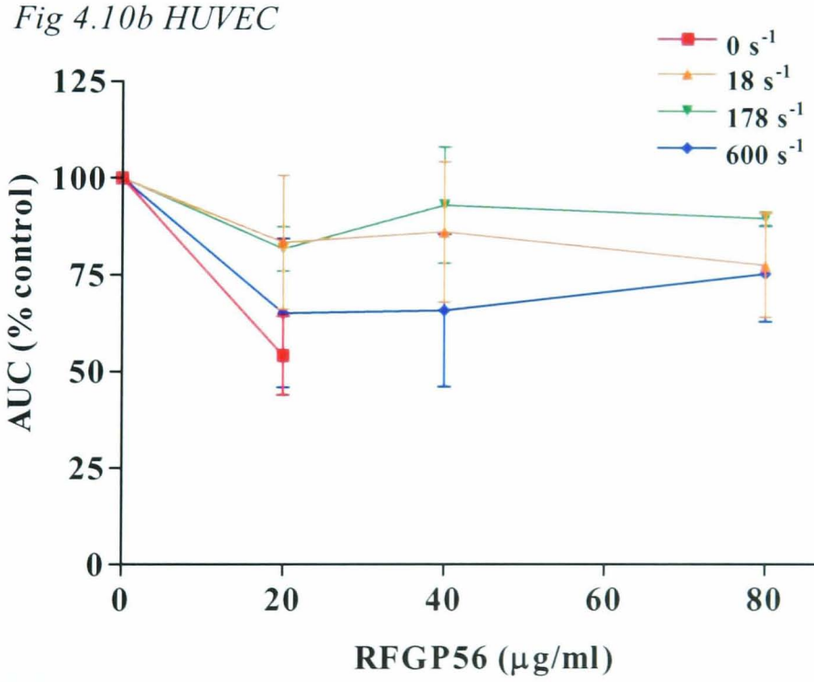


Figure 4.10 *Thrombin generation following extrinsic stimulation with low TF under flow conditions in the presence of HUVEC and RFGP56.*

800 μl of defibrinated plasma containing 300 x 10^6 platelets was incubated for 30 minutes at 37 °C with 25 μl of TBS containing the antibody under test. Thrombin generation was initiated with 175 μl of 100 mM $CaCl_2$ containing recombinant tissue factor to give a final concentration of 35 pM. Timed subsamples were taken into EDTA stop buffer for determination of thrombin concentration with chromogenic substrate S2238.

a) Representative thrombin generation curves from a single experiment and the effect of RFGP56 on b) AUC, c) peak thrombin and d) time of peak thrombin.

Fig 4.10c HUVEC

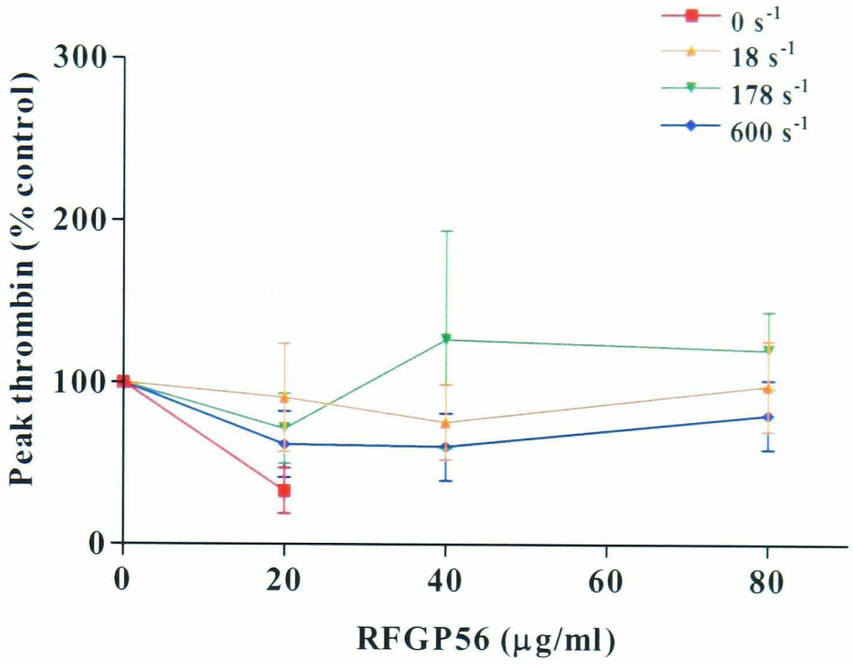


Fig 4.10d HUVEC

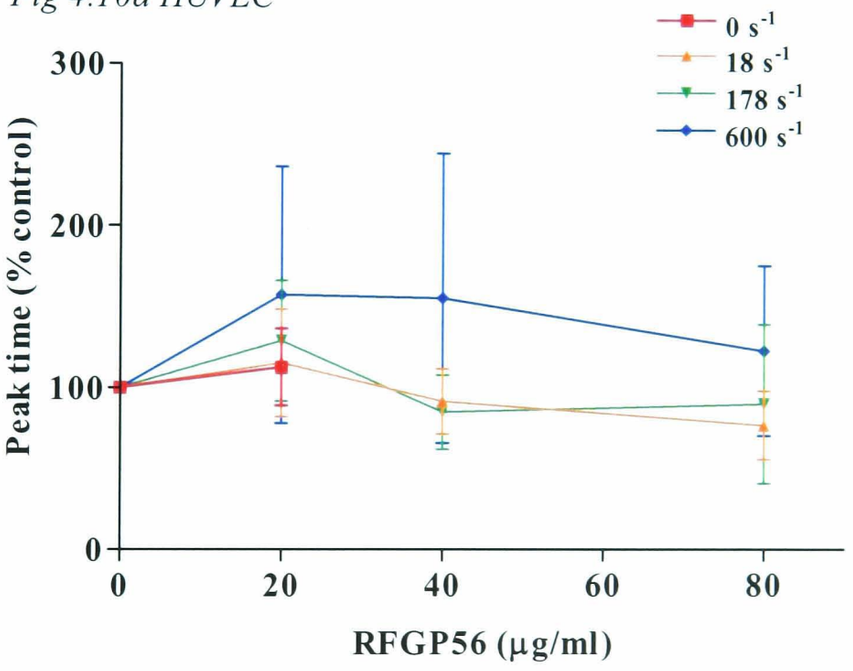


Figure 4.10 *Thrombin generation following extrinsic stimulation with low TF under flow conditions in the presence of HUVEC and RFGP56.*

800 µl of defibrinated plasma containing 300 x 10^6 platelets was incubated for 30 minutes at 37 °C with 25 µl of TBS containing the antibody under test. Thrombin generation was initiated with 175 µl of 100 mM $CaCl_2$ containing recombinant tissue factor to give a final concentration of 35 pM. Timed subsamples were taken into EDTA stop buffer for determination of thrombin concentration with chromogenic substrate S2238.

a) Representative thrombin generation curves from a single experiment and the effect of RFGP56 on b) AUC, c) peak thrombin and d) time of peak thrombin.

Fig 4.11a ECM + RFGP56

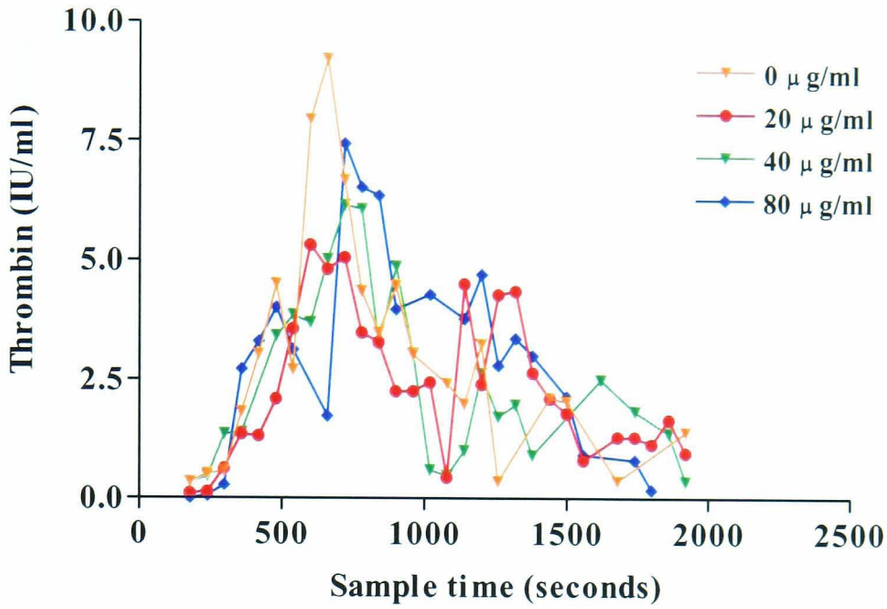


Fig 4.11b ECM

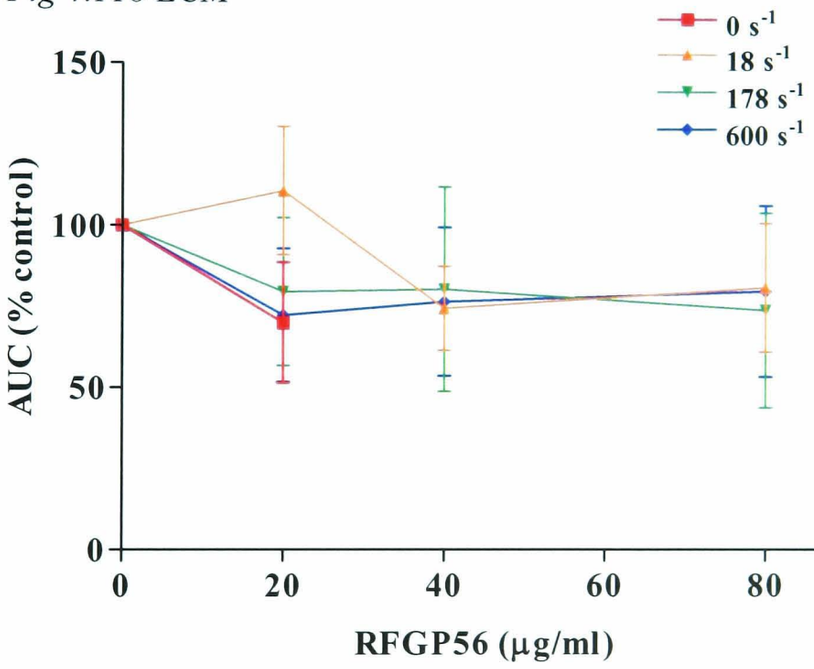


Figure 4.11 *Thrombin generation following extrinsic stimulation with low TF under flow conditions in the presence of ECM and RFGP56.*

800 μl of defibrinated plasma containing 300 x 10^6 platelets was incubated for 30 minutes at 37 °C with 25 μl of TBS containing the antibody under test. Thrombin generation was initiated with 175 μl of 100 mM $CaCl_2$ containing recombinant tissue factor to give a final concentration of 35 pM. Timed subsamples were taken into EDTA stop buffer for determination of thrombin concentration with chromogenic substrate S2238.

a) Representative thrombin generation curves from a single experiment and the effect of RFGP56 on b) AUC, c) peak thrombin and d) time of peak thrombin.

Fig 4.11c ECM

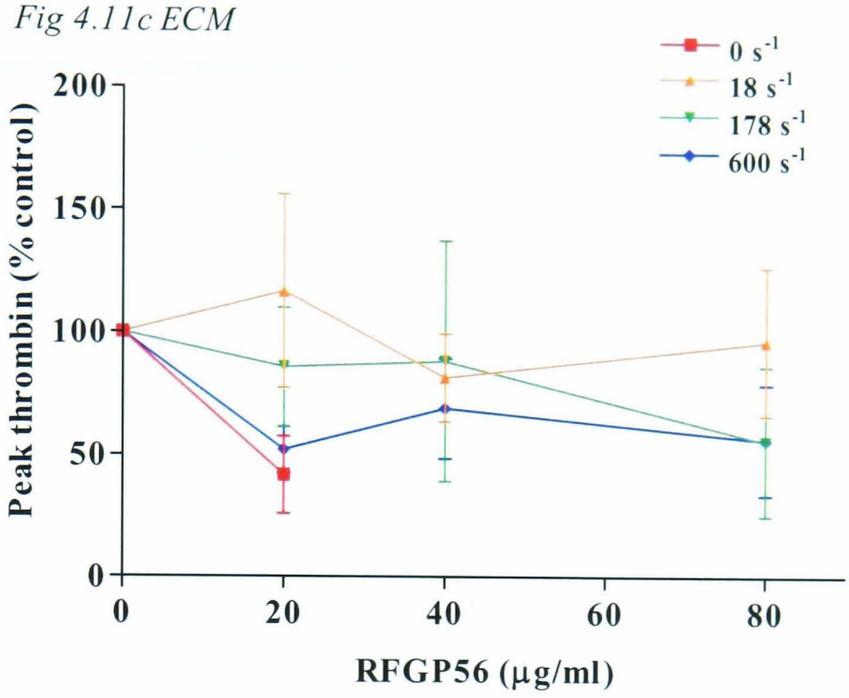


Fig 4.11d ECM

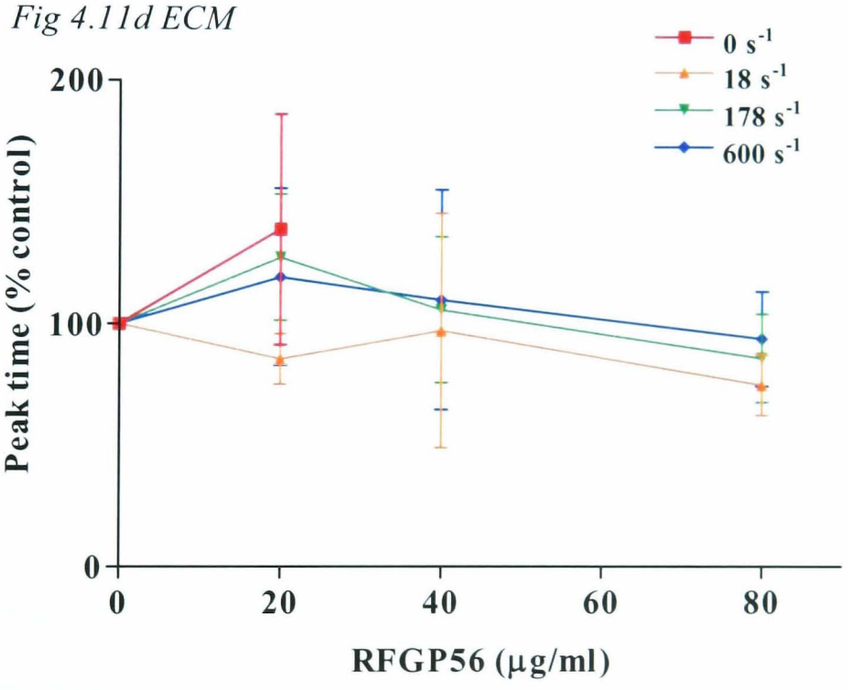


Figure 4.11 *Thrombin generation following extrinsic stimulation with low TF under flow conditions in the presence of ECM and RFGP56.*

800 µl of defibrinated plasma containing 300 x 10^6 platelets was incubated for 30 minutes at 37 °C with 25 µl of TBS containing the antibody under test. Thrombin generation was initiated with 175 µl of 100 mM $CaCl_2$ containing recombinant tissue factor to give a final concentration of 35 pM. Timed subsamples were taken into EDTA stop buffer for determination of thrombin concentration with chromogenic substrate S2238.

a) Representative thrombin generation curves from a single experiment and the effect of RFGP56 on b) AUC, c) peak thrombin and d) time of peak thrombin.

4.3.6.2 RFGP37

When thrombin generation was measured under static conditions in the presence or absence of HUVEC, there was no significant difference in the inhibitory effect of Cymbus RFGP37. Twenty μg/ml Cymbus RFGP37 inhibited AUC to 73.5 ± 26.8 % of control values in the presence of HUVEC, compared to 81.0 ± 1.3 % in the absence of HUVEC in polystyrene test tubes (as reported in Section 4.3.5, using the clotting method of thrombin determination). The introduction of shear stress had no significant effect on inhibition in the presence of HUVEC by 20 μg/ml Cymbus RFGP37, which remained at 88.9 ± 12.9, 85.8 ± 9.8 and 80.0 (n = 1) % of control AUC at 18, 178 and 600 s^{-1} respectively (main panel of Figure 4.12b). Increasing the concentration of Cymbus RFGP37 up to 80 μg/ml appeared to increase the inhibitory effect, although an insufficient number of repeats were performed before the antibody stock was exhausted. At 178 and 600 s^{-1}, 80 μg/ml Cymbus RFGP37 reduced thrombin generation to 47.1 ± 14.3 and 35.9 ± 3.9 % respectively (n = 2). Over the range of concentrations tested, ANOVA revealed that in the presence of HUVEC, Cymbus RFGP37 was a significantly better inhibitor of AUC under arterial conditions (600 s^{-1}) compared with venous (18 s^{-1}). No significant differences were seen in peak thrombin generation or the peak time (main panels of Figure 4.12c and d, respectively).

In the presence of ECM, thrombin generation under static conditions was unaffected (98.4 ± 10.7 %) by 20 μg/ml Cymbus RFGP37 (Figure 4.13a). The introduction of shear stress in the presence of ECM led to some differences, with AUC of 105.9 ± 3.7, 81.4 ± 1.8 and 112.0 ± 0.7 at 18, 178 and 600 s^{-1} respectively ($p < 0.01$ in all cases, but n = 2; Figure 4.13b). Increasing the concentration of Cymbus

RFGP37 up to 80 μg/ml led to significant inhibition, with AUC of 59.9 ± 28.6 and 54.5 ± 16.2 % at 178 and 600 s^{-1} respectively ($p < 0.01$). ANOVA revealed that Cymbus RFGP37 was a significantly better inhibitor of AUC under venous conditions (178 s^{-1}) than static conditions ($p < 0.05$). No significant differences were seen in peak thrombin generation or the peak time (main panels of Figure 4.13c and d respectively).

Serotec RFGP37 had no significant effect ($n \geq 3$) on any of the parameters of thrombin generation in the presence of either HUVEC or ECM at any of the concentrations tested. It was tested at shear rates of 178 and 600 s^{-1}, following exhaustion of the original stock of Cymbus RFGP37 (see inset panels in Figures 4.12 and 4.13).

Fig 4.12a HUVEC + Cymbus RFGP37

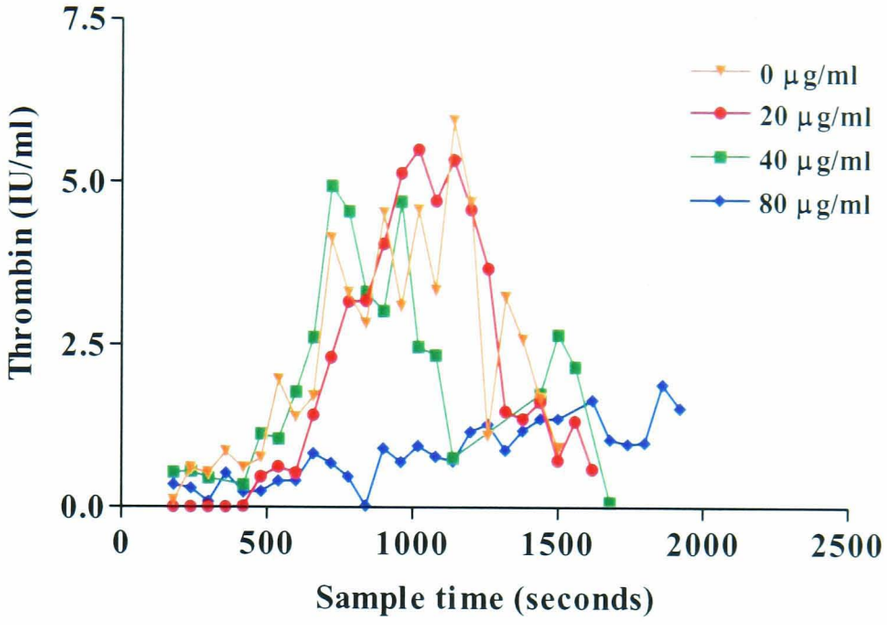


Fig 4.12b HUVEC

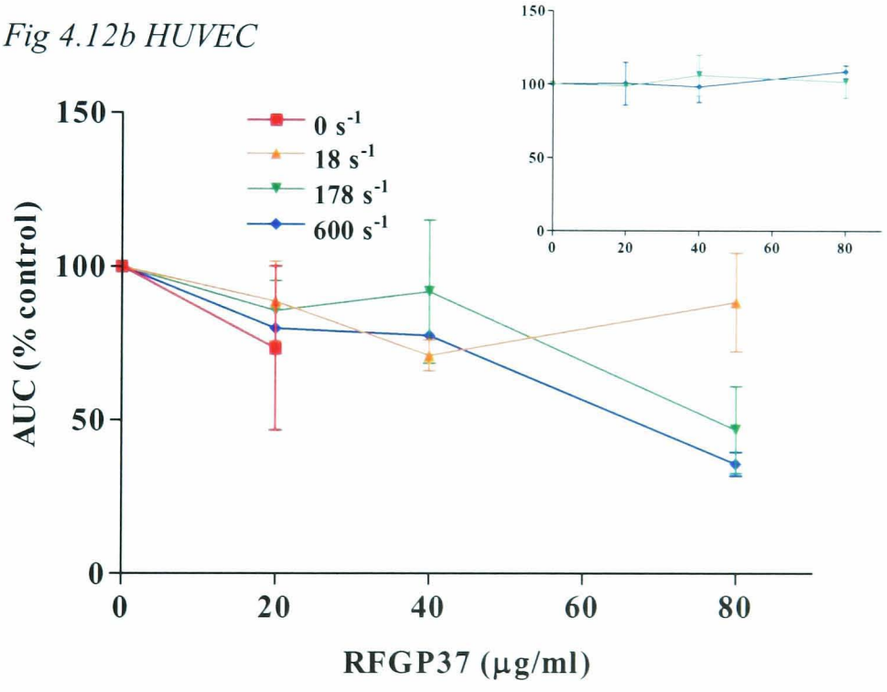


Figure 4.12 *Thrombin generation following extrinsic stimulation with low TF under flow conditions in the presence of HUVEC and RFGP37.*

800 µl of defibrinated plasma containing 300 x 10^6 platelets was incubated for 30 minutes at 37 °C with 25 µl of TBS containing the antibody under test. Thrombin generation was initiated with 175 µl of 100 mM $CaCl_2$ containing recombinant tissue factor to give a final concentration of 35 pM. Timed subsamples were taken into EDTA stop buffer for determination of thrombin concentration with chromogenic substrate S2238.

a) Representative thrombin generation curves from a single experiment and the effect of Cymbus RFGP37 on b) AUC, c) peak thrombin and d) time of peak thrombin. Inset is Serotec RFGP37.

Fig 4.12c HUVEC

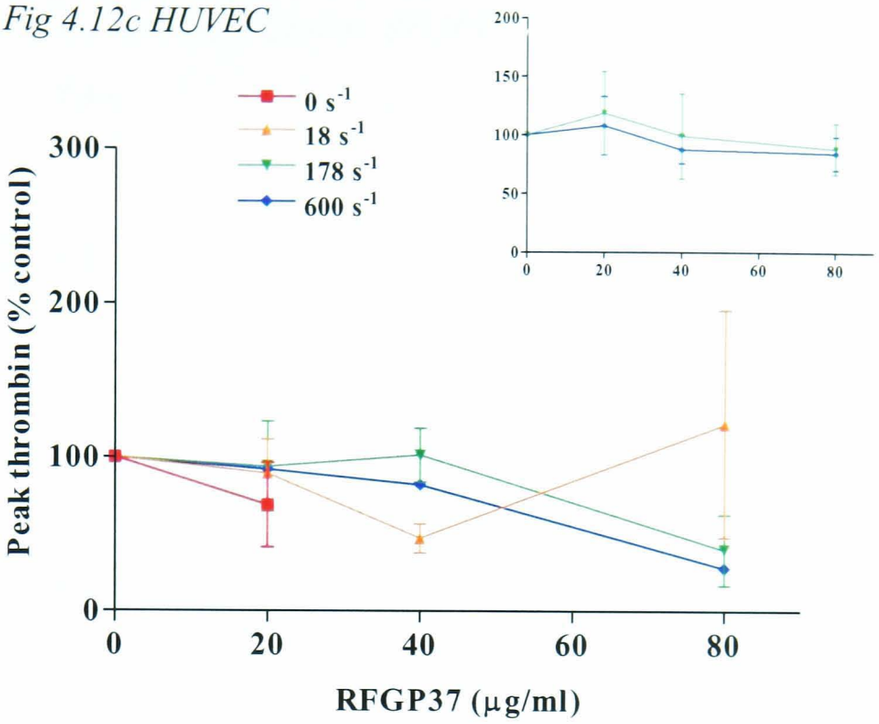


Fig 4.12d HUVEC

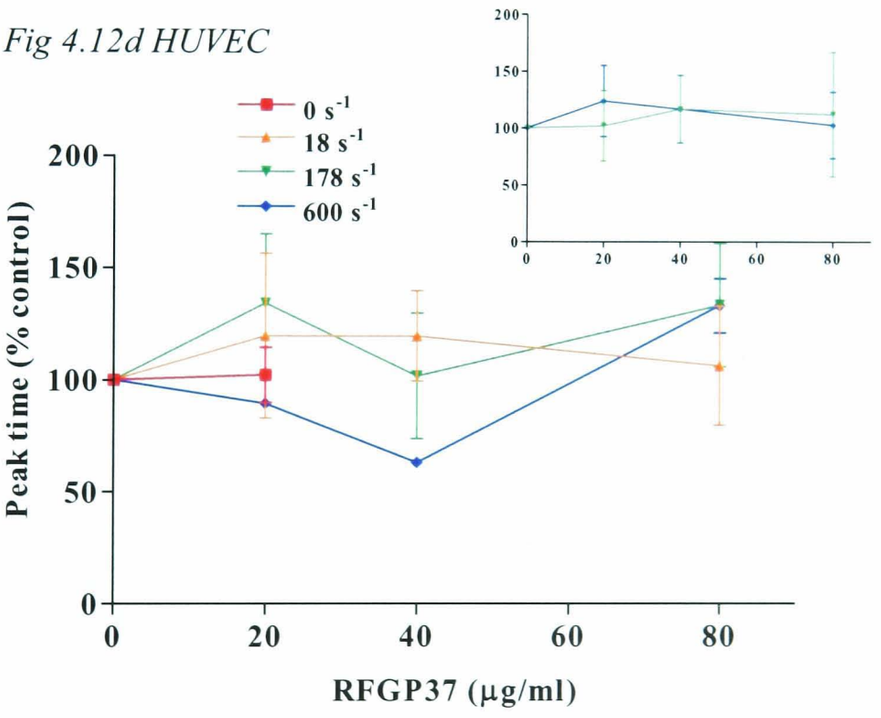


Figure 4.12 *Thrombin generation following extrinsic stimulation with low TF under flow conditions in the presence of HUVEC and RFGP37.*

800 µl of defibrinated plasma containing 300 x 10^6 platelets was incubated for 30 minutes at 37 °C with 25 µl of TBS containing the antibody under test. Thrombin generation was initiated with 175 µl of 100 mM $CaCl_2$ containing recombinant tissue factor to give a final concentration of 35 pM. Timed subsamples were taken into EDTA stop buffer for determination of thrombin concentration with chromogenic substrate S2238.

a) Representative thrombin generation curves from a single experiment and the effect of Cymbus RFGP37 on b) AUC, c) peak thrombin and d) time of peak thrombin. Inset is Serotec RFGP37.

Fig 4.13a ECM + Cymbus RFGP37

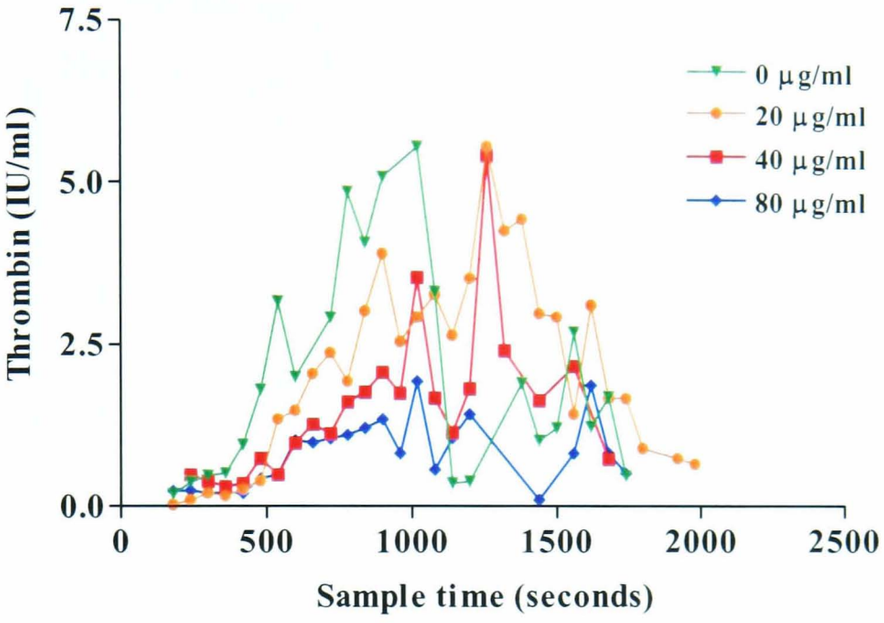


Fig 4.13b ECM

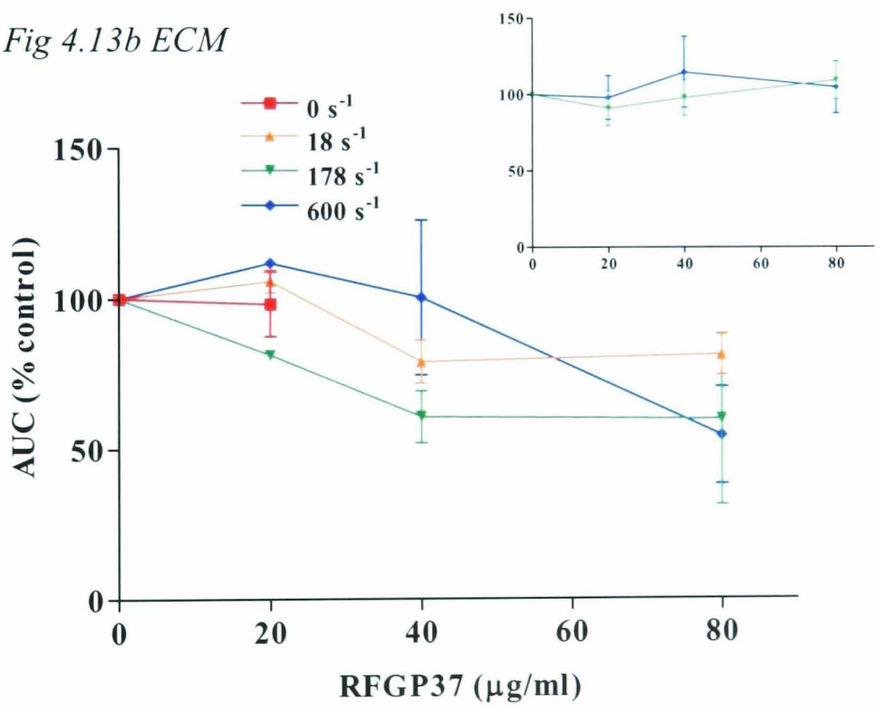


Figure 4.13 *Thrombin generation following extrinsic stimulation with low TF under flow conditions in the presence of ECM and RFGP37.*

800 µl of defibrinated plasma containing 300 x 10^6 platelets was incubated for 30 minutes at 37 °C with 25 µl of TBS containing the antibody under test. Thrombin generation was initiated with 175 µl of 100 mM $CaCl_2$ containing recombinant tissue factor to give a final concentration of 35 pM. Timed subsamples were taken into EDTA stop buffer for determination of thrombin concentration with chromogenic substrate S2238.

a) Representative thrombin generation curves from a single experiment and the effect of Cymbus RFGP37 on b) AUC, c) peak thrombin and d) time of peak thrombin. Inset is Serotec RFGP37.

Fig 4.13c ECM

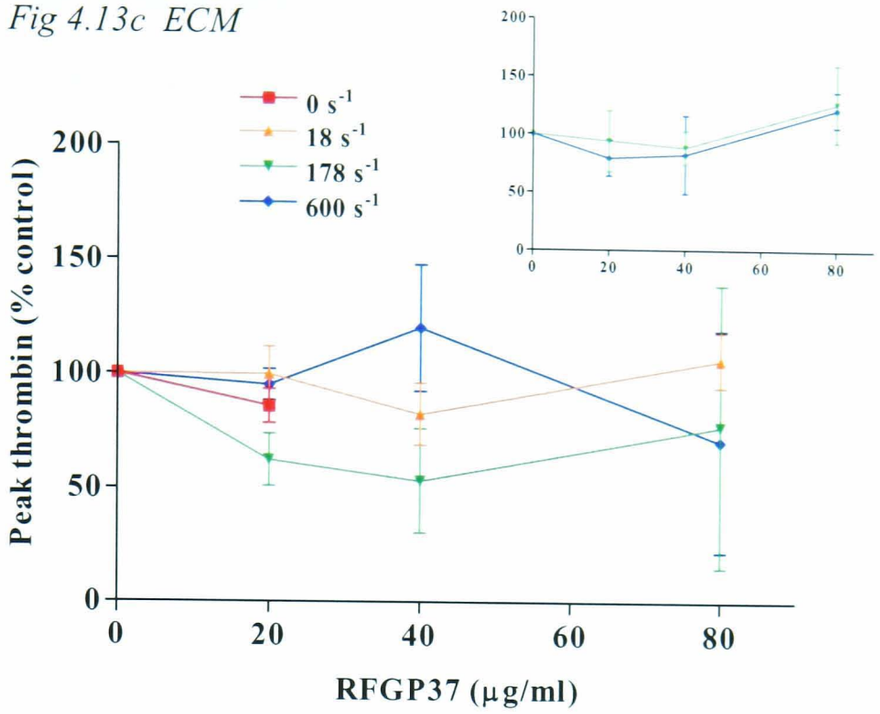


Fig 4.13d ECM

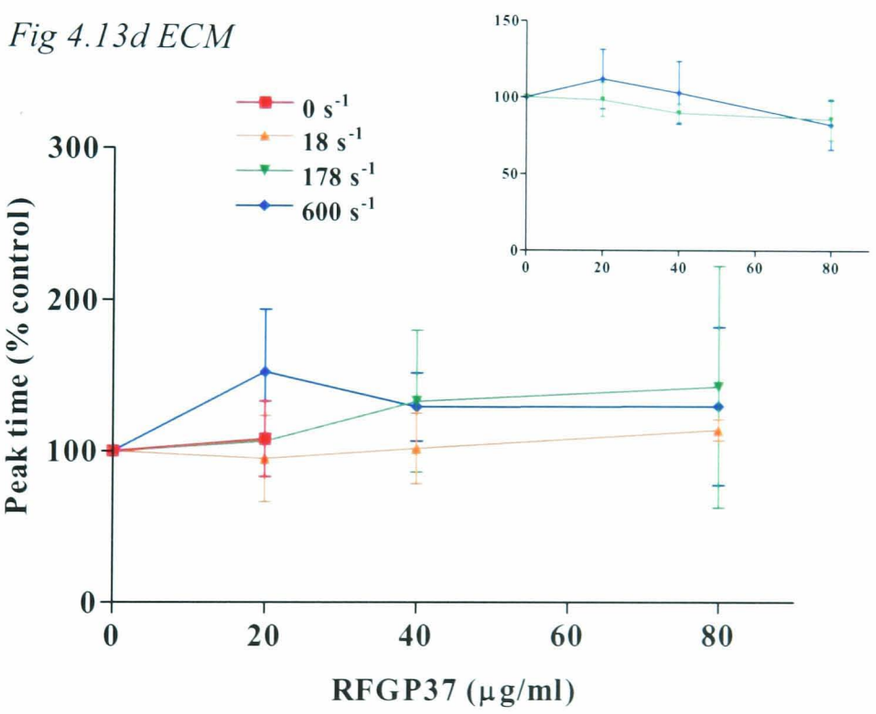


Figure 4.13 *Thrombin generation following extrinsic stimulation with low TF under flow conditions in the presence of ECM and RFGP37.*

800 µl of defibrinated plasma containing 300 x 10^6 platelets was incubated for 30 minutes at 37 °C with 25 µl of TBS containing the antibody under test. Thrombin generation was initiated with 175 µl of 100 mM $CaCl_2$ containing recombinant tissue factor to give a final concentration of 35 pM. Timed subsamples were taken into EDTA stop buffer for determination of thrombin concentration with chromogenic substrate S2238.

a) Representative thrombin generation curves from a single experiment and the effect of Cymbus RFGP37 on b) AUC, c) peak thrombin and d) time of peak thrombin. Inset is Serotec RFGP37.

4.3.6.3 RFGP56 and RFGP37 in combination

When thrombin generation was measured under flow conditions in the presence of HUVEC (Figure 4.14), the combination of 20 μg/ml each of RFGP56 and Cymbus RFGP37 significantly inhibited AUC to 62.2 ± 6.8 % ($p < 0.01$) at 18 s^{-1}. No further effects were seen at concentrations up to 80 μg/ml, and similar results were seen at 178 s^{-1} ($n = 2$) and 600 s^{-1} ($n = 1$). No significant differences could be detected by ANOVA in AUC, peak thrombin or time of peak (main panels of Figures 4.14b, c and d, respectively).

The combination of RFGP56 and Serotec RFGP37 at 20 μg/ml each in the presence of HUVEC inhibited AUC under static conditions to 64.1 ± 13.5 % ($p < 0.01$), with no further effect at concentrations up to 80 μg/ml (Figure 4.14b inset). ANOVA revealed that the combination of RFGP56 and Serotec RFGP37 was significantly more effective in reducing the AUC under static and arterial (600 s^{-1}) conditions than venous conditions (178 s^{-1}). However, in terms of peak height, this combination was significantly more effective under static conditions than venous (178 s^{-1}) or arterial (600 s^{-1}) conditions (Figure 4.14c inset). No significant differences were seen in terms of peak time (Figure 4.14d inset).

When thrombin generation was measured under flow conditions in the presence of ECM (Figure 4.15), the combination of 20 μg/ml of both RFGP56 and Cymbus RFGP37 significantly inhibited AUC to 73.2 ± 14.8, 58.5 ± 14.6 and 37.9 ± 11.3 % ($p < 0.05$, 0.01, 0.01 respectively) at 18 s^{-1}. Similar, but non-significant, results were seen at 600 s^{-1} ($n = 2$). No significant differences could be detected by ANOVA in

AUC, peak thrombin or time of peak (main panels of Figures 4.15b, c and d, respectively).

The combination of RFGP56 and Serotec RFGP37 at concentrations as high as 80 μg/ml each in the presence of HUVEC had no significant effect on any of the parameters measured ($n \geq 3$; see inset panels in Figures 4.14 and 4.15).

Fig 4.14a HUVEC + RFGP56 + Cymbus RFGP37; 600 s^{-1}

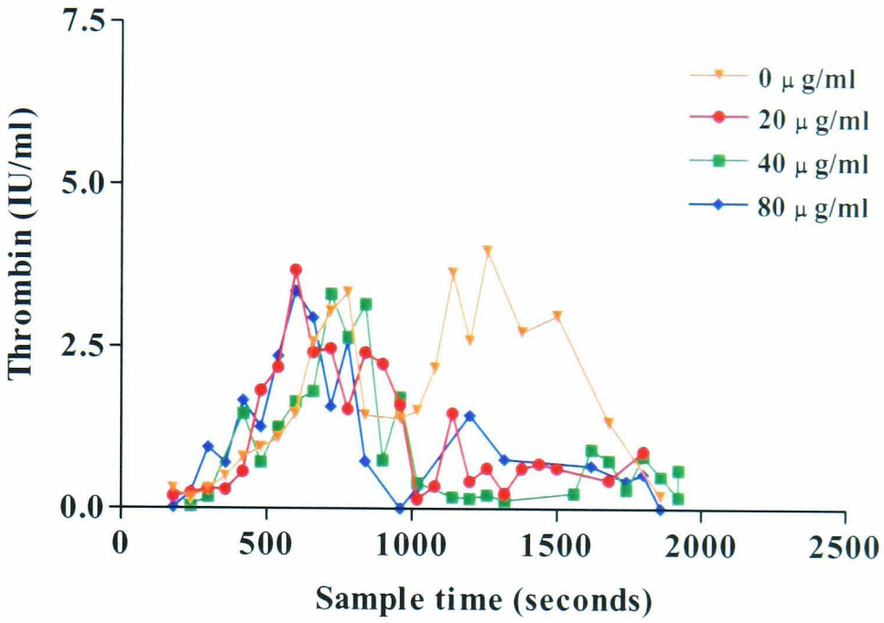


Fig 4.14b HUVEC

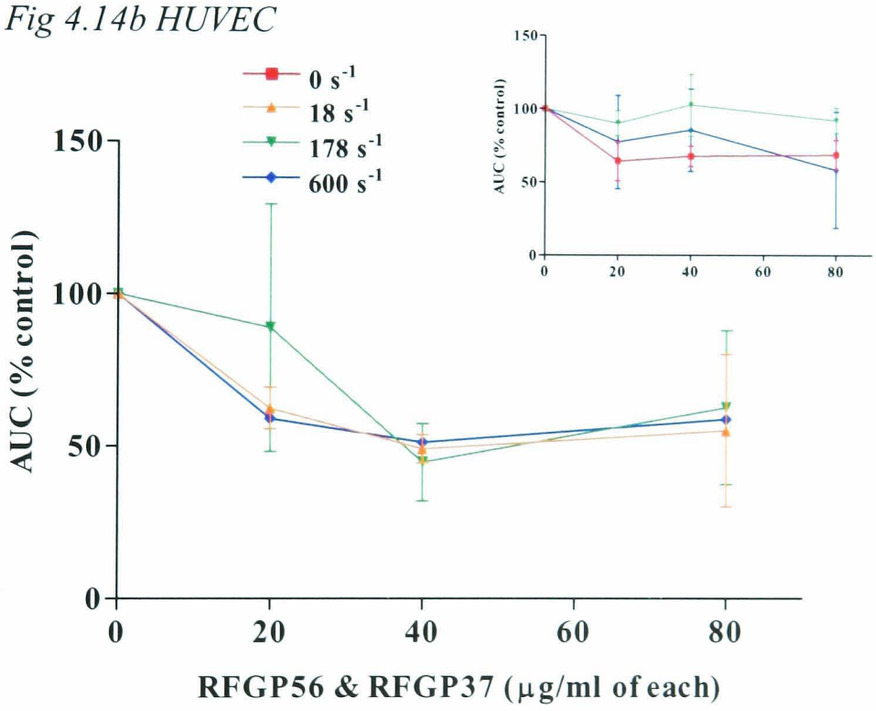
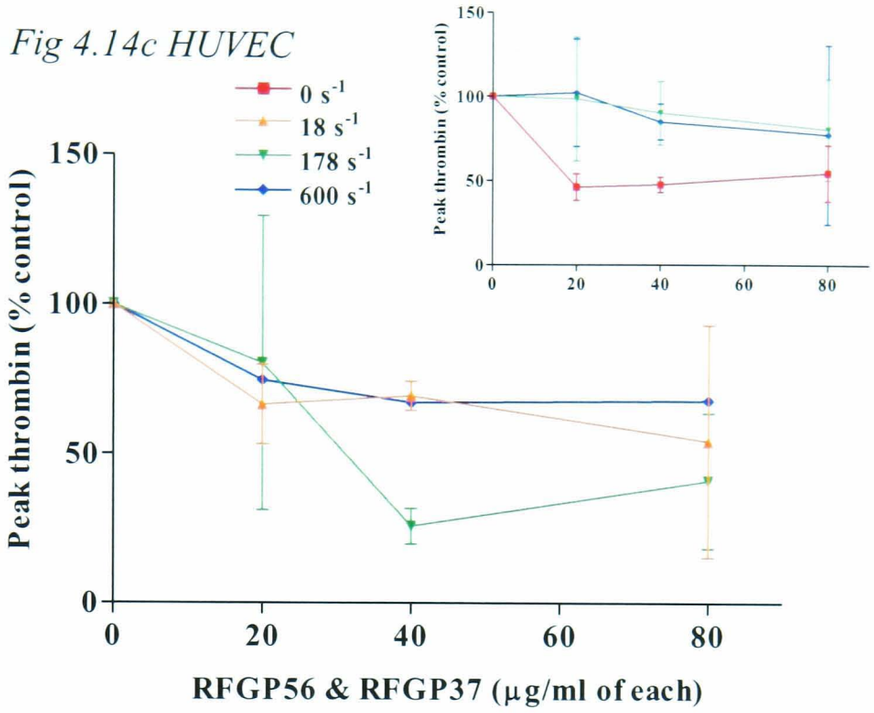


Figure 4.14 *Thrombin generation following extrinsic stimulation with low TF under flow conditions in the presence of HUVEC, RFGP56 and RFGP37.*

800 μl of defibrinated plasma containing 300 x 10^6 platelets was incubated for 30 minutes at 37 °C with 25 μl of TBS containing the antibody under test. Thrombin generation was initiated with 175 μl of 100 mM $CaCl_2$ containing recombinant tissue factor to give a final concentration of 35 pM. Timed subsamples were taken into EDTA stop buffer for determination of thrombin concentration with chromogenic substrate S2238.

a) Representative thrombin generation curves from a single experiment and the effect of RFGP56 + Cymbus RFGP37 on b) AUC, c) peak thrombin and d) time of peak thrombin. Inset is RFGP56 + Serotec RFGP37.



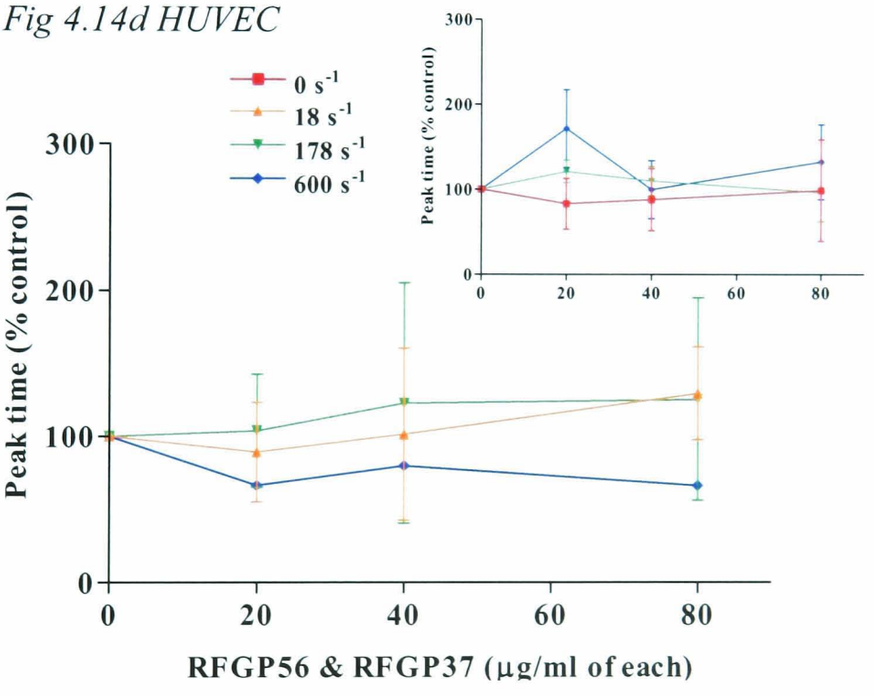


Figure 4.14 *Thrombin generation following extrinsic stimulation with low TF under flow conditions in the presence of HUVEC, RFGP56 and RFGP37.*

800 µl of defibrinated plasma containing 300 x 10^6 platelets was incubated for 30 minutes at 37 °C with 25 µl of TBS containing the antibody under test. Thrombin generation was initiated with 175 µl of 100 mM $CaCl_2$ containing recombinant tissue factor to give a final concentration of 35 pM. Timed subsamples were taken into EDTA stop buffer for determination of thrombin concentration with chromogenic substrate S2238.

a) Representative thrombin generation curves from a single experiment and the effect of RFGP56 + Cymbus RFGP37 on b) AUC, c) peak thrombin and d) time of peak thrombin. Inset is RFGP56 + Serotec RFGP37.

Fig 4.15a ECM + RFGP56 + Cymbus RFGP37; 600 s^{-1}

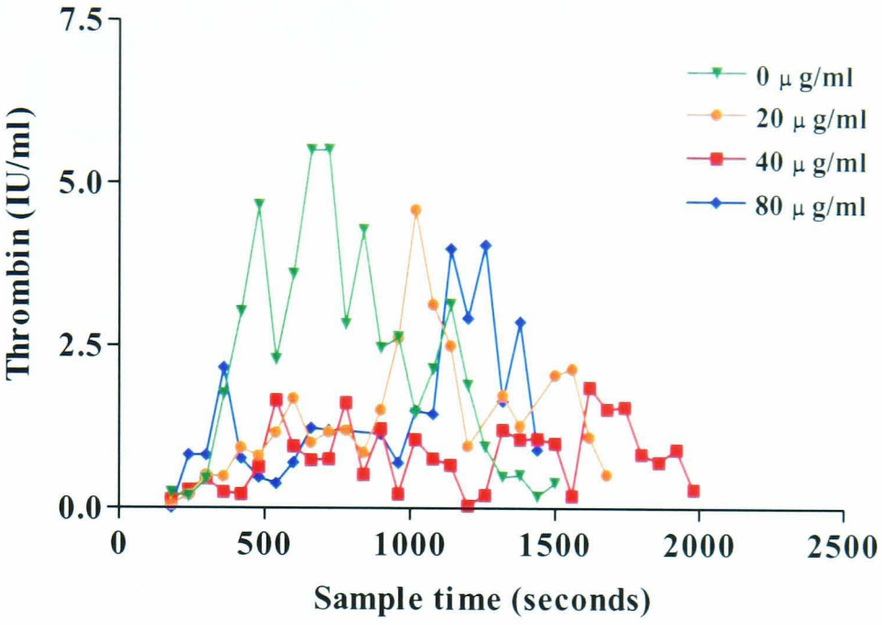


Fig 4.15b ECM

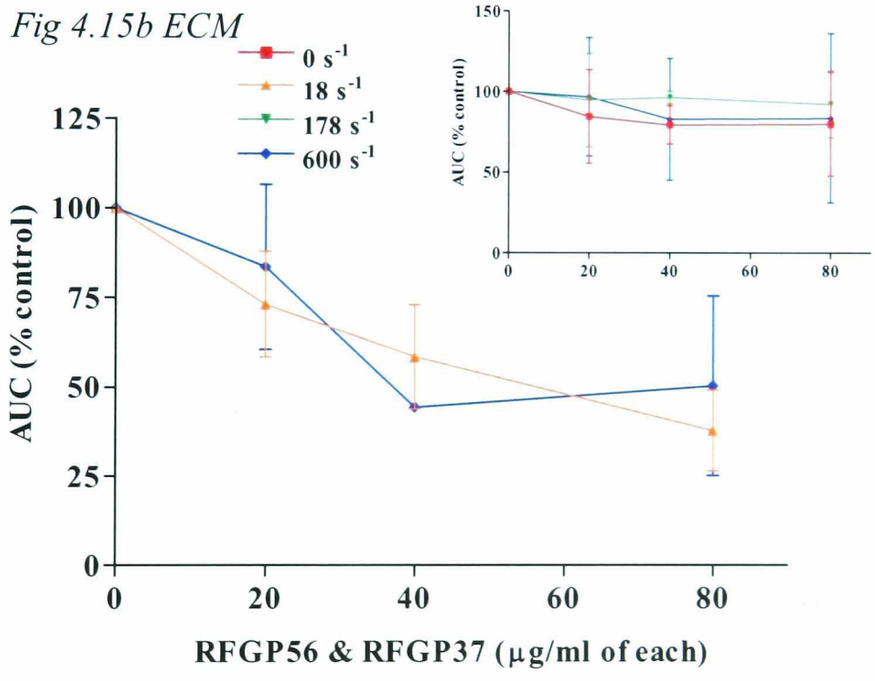


Figure 4.15 *Thrombin generation following extrinsic stimulation with low TF under flow conditions in the presence of ECM, RFGP56 and RFGP37.*

800 μl of defibrinated plasma containing 300 x 10^6 platelets was incubated for 30 minutes at 37 °C with 25 μl of TBS containing the antibody under test. Thrombin generation was initiated with 175 μl of 100 mM $CaCl_2$ containing recombinant tissue factor to give a final concentration of 35 pM. Timed subsamples were taken into EDTA stop buffer for determination of thrombin concentration with chromogenic substrate S2238.

a) Representative thrombin generation curves from a single experiment and the effect of RFGP56 + Cymbus RFGP37 on b) AUC, c) peak thrombin and d) time of peak thrombin. Inset is RFGP56 + Serotec RFGP37.

Fig 4.15c ECM

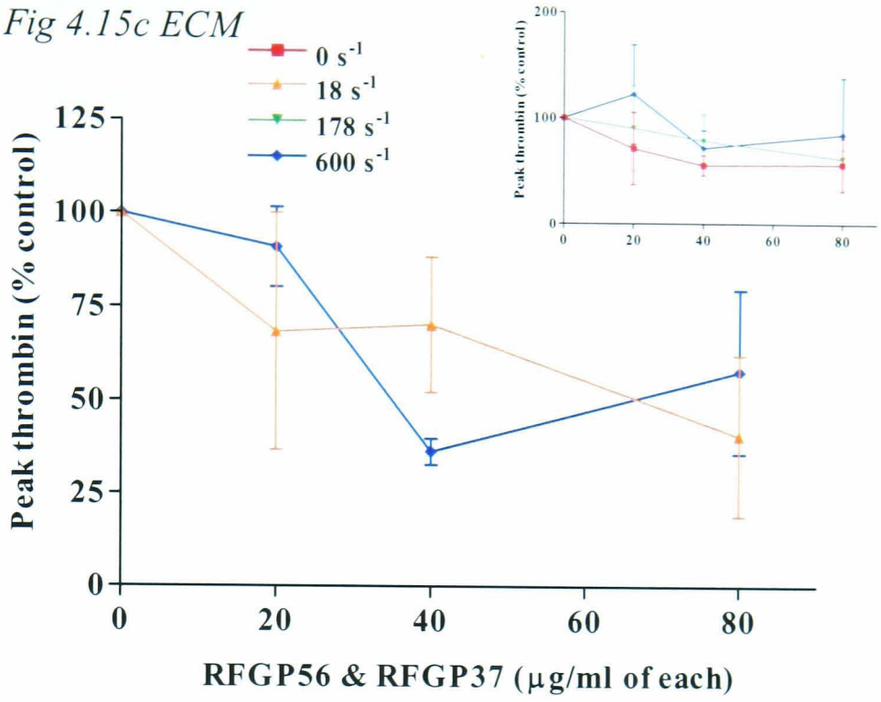


Fig 4.15d ECM

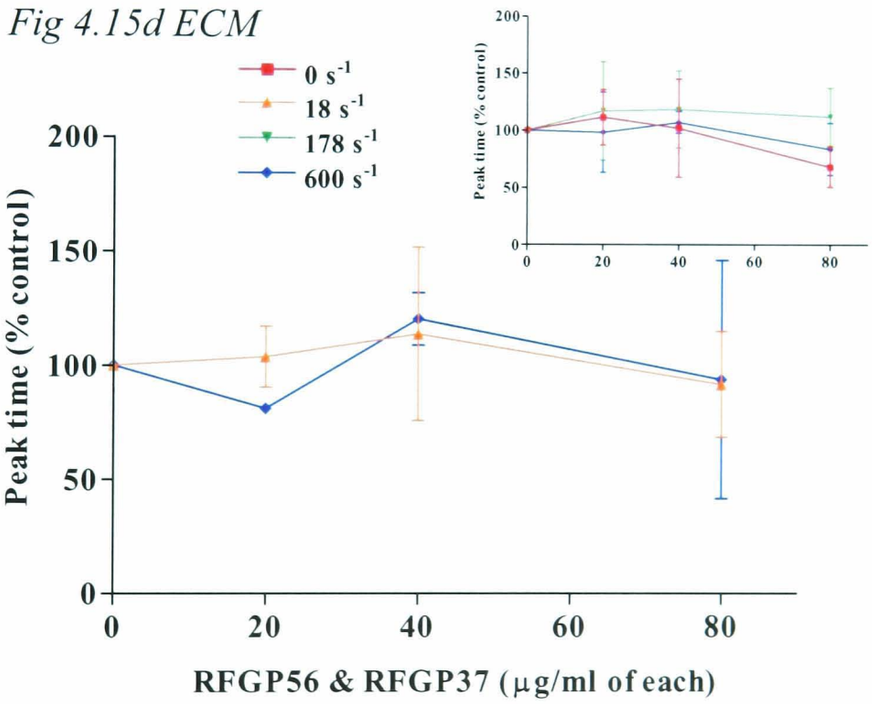


Figure 4.15 *Thrombin generation following extrinsic stimulation with low TF under flow conditions in the presence of ECM, RFGP56 and RFGP37.*

800 µl of defibrinated plasma containing 300 x 10^6 platelets was incubated for 30 minutes at 37 ºC with 25 µl of TBS containing the antibody under test. Thrombin generation was initiated with 175 µl of 100 mM $CaCl_2$ containing recombinant tissue factor to give a final concentration of 35 pM. Timed subsamples were taken into EDTA stop buffer for determination of thrombin concentration with chromogenic substrate S2238.

a) Representative thrombin generation curves from a single experiment and the effect of RFGP56 + Cymbus RFGP37 on b) AUC, c) peak thrombin and d) time of peak thrombin. Inset is RFGP56 + Serotec RFGP37.

4.3.7 Expression of negatively charged phospholipids

Flow cytometry was performed to detect FITC-annexin-5A binding to unstimulated and TF stimulated platelets following incubation with 20 μg/ml of antibody. The data obtained were analysed to determine the percentage of platelets positive for annexin-5A binding (Figure 4.16).

Annexin-5A binding to platelets stimulated with TF was reduced by the presence of 20 μg/ml of anti-GP IIb/IIIa antibodies. RFGP56 and c7E3 reduced the percentage of annexin-5A positive platelets to 33.3 ± 13.8 % ($p < 0.01$) and 34.7 ± 9.2 % ($p < 0.01$) of control levels respectively (Figure 4.16c). In contrast, the presence of 20 μg/ml of the anti-GP Ibα antibody Cymbus RFGP37 or its $F(ab')_2$ fragment (in order to investigate the possibility of stimulation via the Fc receptor; Cauwenberghs *et al*, 2001) caused increased binding of annexin-5A, to 121.6 ± 16.4 % (ns) and 142.1 ± 19.5 % ($p < 0.01$), respectively. However, when 20 μg/ml of both RFGP56 and Cymbus RFGP37 were present, the overall effect was a reduction in annexin-5a binding to 44.2 ± 6.97 % ($p < 0.01$). No significant effect was seen in the presence of the irrelevant anti-D control antibody, Reg A, at either 20 or 40 μg/ml (Figure 4.16c).

Fig 4.16a

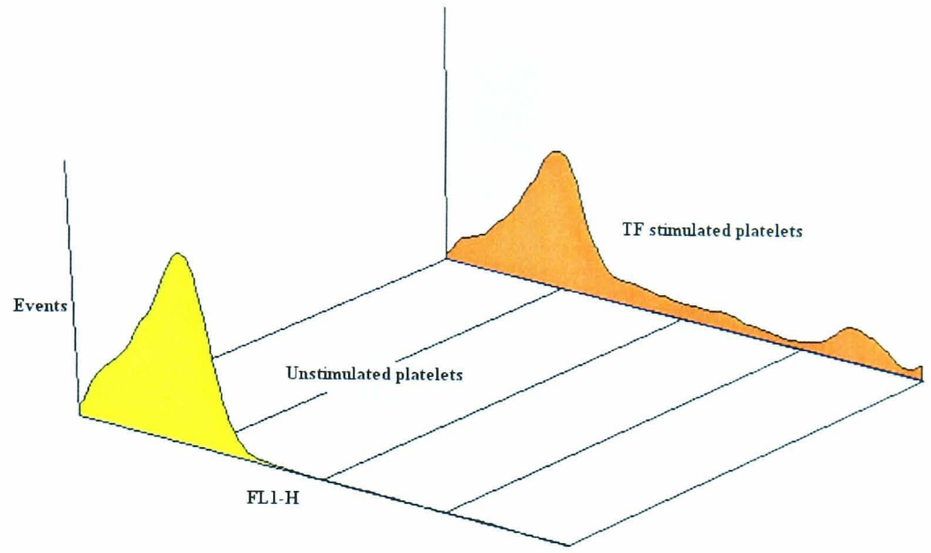


Fig 4.16b

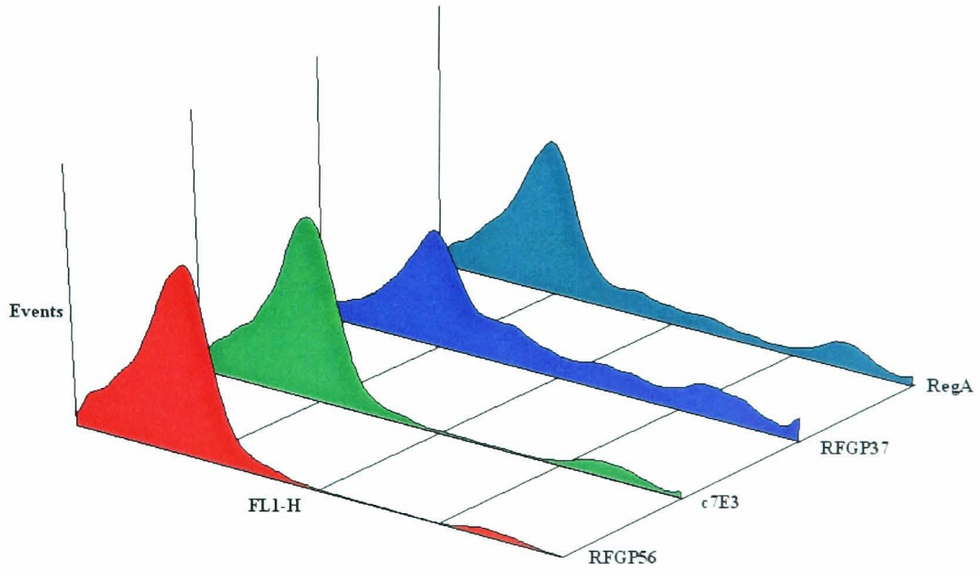


Figure 4.16 *FITC-annexin-5A binding to unstimulated and TF-stimulated platelets.*

a) Defibrinated plasma containing 300 x 10^6 platelets/ml was stimulated with either 17.5 mM calcium alone (unstimulated platelets) or with low TF and 17.5 mM calcium (TF-stimulated platelets). After 12 minutes, a subsample was taken into HBS containing hirudin, a subsample of this mixture was taken into HBS containing FITC-Annexin-5A and 10 000 events counted on a flow cytometer.

b) As for a), but platelets were incubated with 20 µg/ml of antibody for 20 minutes prior to stimulation with low TF and 17.5 mM calcium.

Fig 4.16c

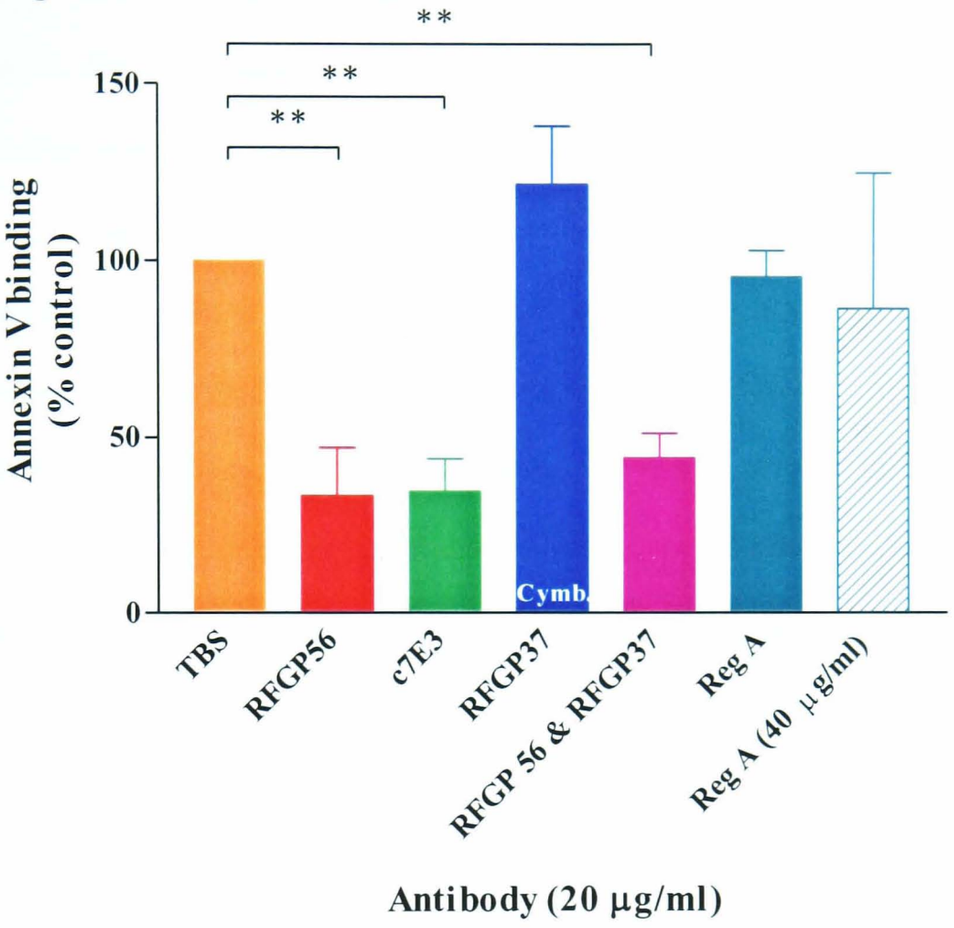


Figure 4.16 *FITC-annexin-5A binding to unstimulated and TF-stimulated platelets.*

*c) For each antibody treatment, the difference in the percentage of positive platelets between the TF-stimulated cells and the unstimulated cells was calculated and compared with the control (TBS treated) cells. $n \geq 3$; ** $p < 0.01$*

4.3.8 Measurement of soluble fibrin in defibrinated plasma

Soluble fibrin was easily detected in plasma that had been defibrinated with ancrod, and little difference was seen between 0.5 and 1.0 IU/ml of ancrod for 20 minutes at 37 °C (Figure 4.17 main panel). No soluble fibrin was detected following immunoprecipitation with the ELISA capture antibody 5F3 after defibrination with ancrod. Plasma that had been defibrinated with batroxobin (5 units/ml) also had measurable levels of soluble fibrin (Figure 4.17 inset). Heat defibrination (56 °C for 60 minutes) was highly effective in the removal of soluble fibrin (Figure 4.17 main panel), but the potential effects of heat on other clotting factors prevented this being used as a practical alternative method.

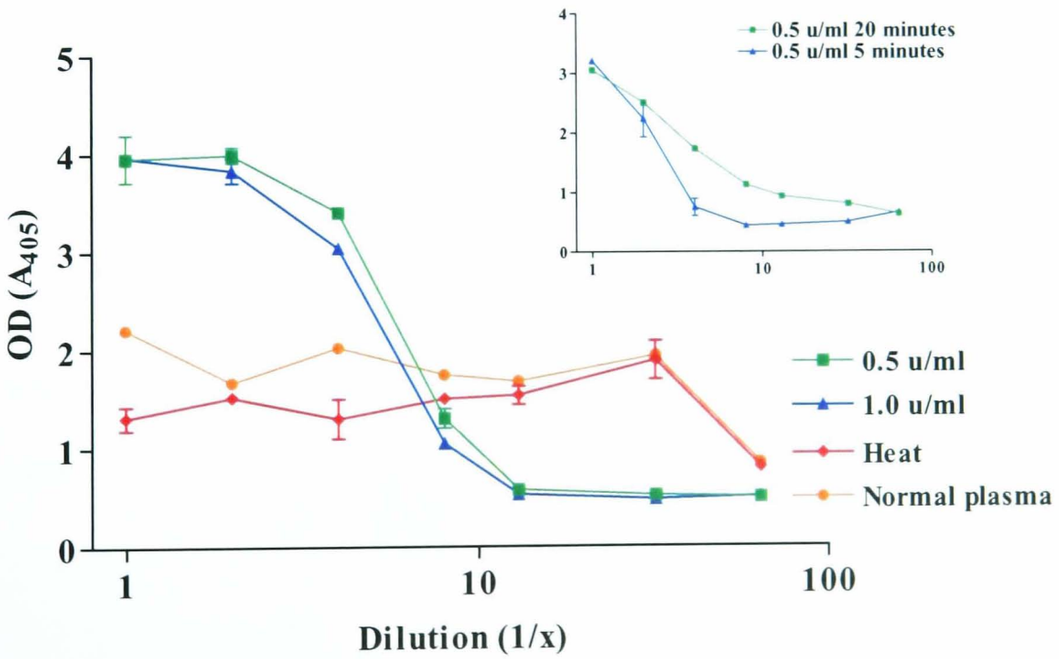


Figure 4.17 *ELISA for detection of soluble fibrin in plasma following defibrination by various methods.*

A microtitre plate was coated with MAb 5F3 and dilutions of the defibrinated plasma (prepared in citrate saline containing GPRP) were added. The secondary antibody was biotinylated MAb A11, the biotin was detected with streptavidin-HRP and o-toluidine (Sigma, Poole, UK) used as the substrate. Main panel shows ancrod data and heat data, batroxobin data shown inset. Mean of duplicate determinations ± SEM.

Incubating at 37 °C with 0.5 IU/ml ancrod for longer (50 or 70 minutes) had no further effect compared to 20 minutes incubation, but incubation at 4 °C overnight following 20 minutes at 37 °C reduced soluble fibrin to below detectable levels. However, when plasma defibrinated in this way was used in a thrombin generation test, very low levels of thrombin were detected (585 IU.seconds/ml; Figure 4.18) suggesting that other coagulation factors such as FVIII had been depleted or destroyed by the incubation. This was studied with a one stage clotting assay for FVIII (Austen & Rhymes, 1975) that confirmed a relative potency of 0.31 compared with normal plasma. In order to perform a comparable thrombin generation test, 0.6 IU/ml of recombinant FVIII (96/598, NIBSC, UK) was added back to the defibrinated plasma, resulting in comparable levels of thrombin generation (4582 and 4692 IU.seconds/ml for overnight defibrination + rFVIII and freshly defibrinated plasma, respectively; Figure 4.18).

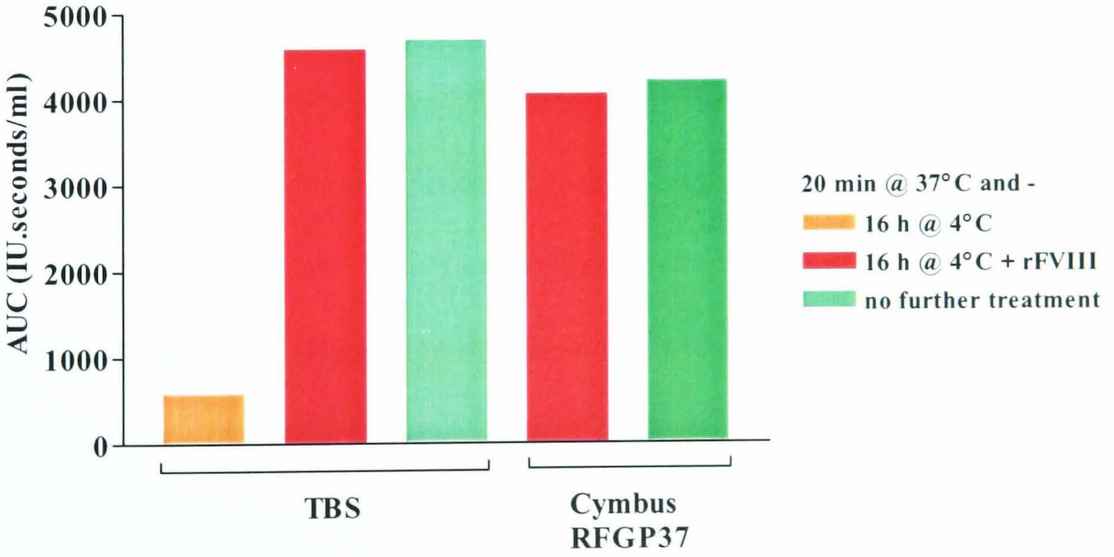


Figure 4.18 *Thrombin generation in various defibrinated plasmas*

Platelets (300 x 10^6/ml) were resuspended in plasma defibrinated by incubation with 0.5 u/ml ancrod at 37 °C for 20 minutes ± 4 °C for 16 hours ± rFVIII. After 30 minutes incubation with 20 μg/ml RFGP37, thrombin generation was triggered under static conditions with 35 pM TF and Ca^{2+} (n = 1). The effect of RFGP37 was studied to determine whether soluble fibrin was stimulating platelets by binding to vWF and enabling it to interact with GP Ibα.

Soluble fibrin in defibrinated plasma may bind to vWF and change its conformation sufficiently to allow vWF to interact with GP Ibα, leading to the activation of the platelet (Béguin *et al*, 1999). RFGP37 binds to the vWF-binding site on GPIbα and this may explain its inhibitory action in thrombin generation (Cox, 1991). In order to test this theory, thrombin generation tests were performed using platelet plasma prepared with plasma defibrinated by incubation at 37 °C for 20 minutes with or without a further 16 hours at 4 °C, with rFVIII added back to the former. As shown in Figure 4.18, the presence of 20 μg/ml Cymbus RFGP37 had no appreciable inhibitory effect in either plasma, suggesting that the presence of soluble fibrin has no influence on the mode of action of RFGP37 in the inhibition of thrombin generation.

Static thrombin generation tests were also performed using RFFVIII:R/1, a monoclonal antibody against the GP Ibα-binding site on vWF that completely inhibited ristocetin-stimulated platelet agglutination at 40 μg/ml. Thrombin generation, measured by AUC, was not significantly inhibited by either 40 μg/ml RFFVIII:R/1 (90.0 ± 9.6 %), or 20 μg/ml Cymbus RFGP37 (89.2 ± 20.5 %), or both antibodies together (97.1 ± 8.7 %; Figure 4.19). If RFGP37 was inhibiting thrombin generation by interfering with a stimulatory pathway involving soluble fibrin, vWF and GP Ibα, it would have an equivalent effect as RFFVIII:R/1, and when tested together, no further inhibition would be seen. RFFVIII:R/1 would also have an additive effect with RFGP56 as they would be operating by inhibiting two distinct pathways. However, inhibition by 20 μg/ml RFGP56 (70.1 ± 14.1 %) was unaffected by the presence of 40 μg/ml RFFVIII:R/1 (71.9 ± 11.4 %).

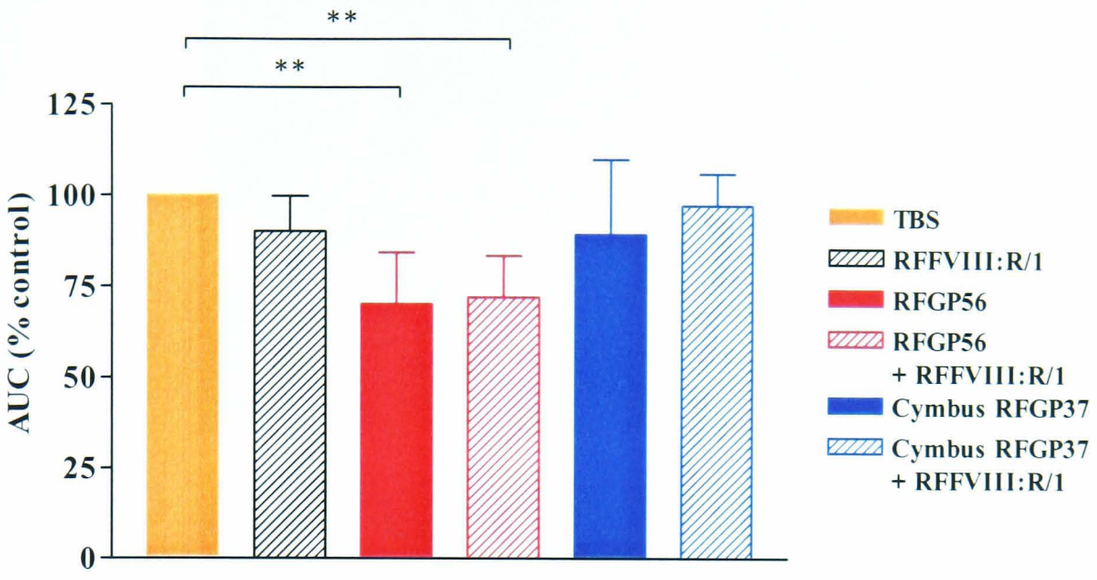


Figure 4.19 *Thrombin generation with anti-vWF monoclonal antibody, RFGP56 and RFGP37.*

RFFVIII:R/1blocks the GP Ibα binding site on vWF and was used to investigate the mode of action of Cymbus RFGP37. 800 µl of defibrinated plasma containing 375 x 10^6 platelets/ml was incubated for 30 minutes at 37 °C with 25 µl of TBS containing RFFVIII:R/1 at 40 µg/ml, RFGP56 and RFGP37 at 20 µg/ml or combinations as indicated. Thrombin generation was initiated with 175 µl of 100 mM $CaCl_2$ containing recombinant tissue factor to give a final concentration of 35 pM. Timed subsamples were taken into EDTA stop buffer for chromogenic determinationof thrombin concentration.

Mean ± standard deviation; $n \geq 5$; $p < 0.01$).

4.3.9 Immunofluorescent staining of platelets and HUVEC

The antiplatelet antibodies RFGP56 and Serotec RFGP37 were clearly seen to bind to platelets, visualised with FITC-rabbit anti-mouse IgG (Figure 4.20). No cross-reactivity was seen with HUVEC, which clearly stained with anti-vWF polyclonal antibody, visualised with FITC-swine anti-rabbit IgG. Platelets did not stain for vWF.

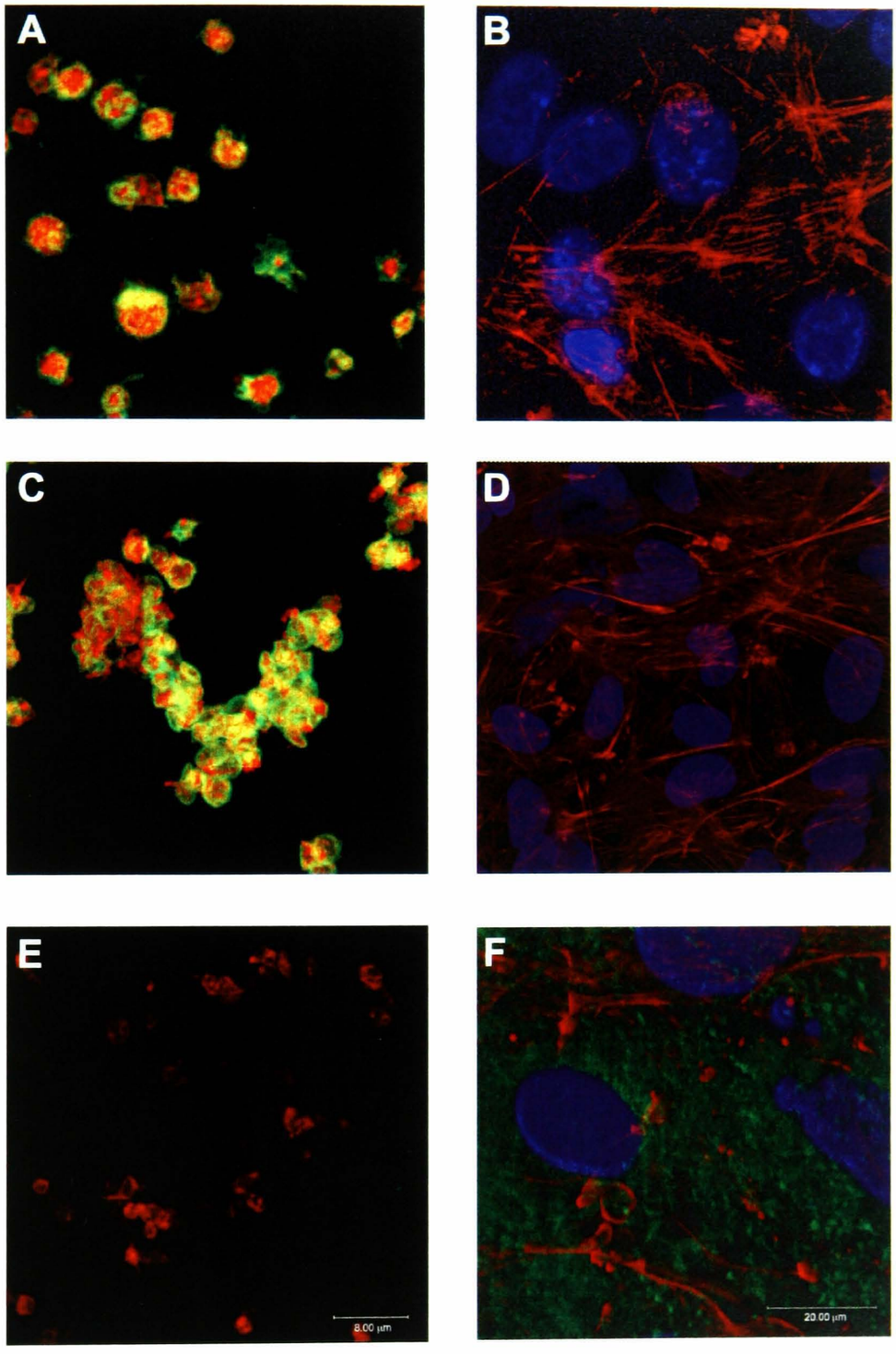


Figure 4.20 *Immunofluorescent staining of antibody binding to platelets and HUVEC.*

Defibrinated plasma containing 375 x 10^6 platelets/ml was incubated with 20 µg/ml of the indicated antibodies for 30 minutes at 37 °C. A drop of this cell suspension was then applied to a glass coverslip treated with poly DL lysine and fixed with 2 % paraformaldehyde. The coverslips were then incubated with 10 µg/ml FITC-secondary antibody (green) to the primary antibodies detailed below and 66 nM Alexa Fluor 532 phalloidin to stain actin (red) in PBS containing 1 % Triton X-100. Nuclei are stained blue following a 10 minute incubation with Hoechst 33342.

A) Platelets + RFGP56

B) HUVEC + RFGP56

C) Platelets + RFGP37

D) HUVEC + RFGP37

E) Platelets + anti-vWF

F) HUVEC + anti-vWF

4.4 Discussion

The effects of antibodies against platelet membrane glycoproteins (GP) IIb/IIIa and Ibα on thrombin generation were studied under static and flow conditions in the presence of intact endothelial cells or their extracellular matrix. Further investigations were undertaken in order to elucidate the mode of action of these antibodies.

GP IIb/IIIa acts as a receptor for fibrinogen, vWF, vitronectin and fibronectin, and as such plays a number of roles in the coagulation process. In resting platelets GP IIb/IIIa is expressed in a low affinity form that only becomes activated enabling high affinity binding to ligands following stimulation of the platelet. Suitable stimuli include ADP, collagen and thrombin, all of which are found at sites of vessel injury. Following platelet activation, the increased affinity of GP IIb/IIIa to bind the multivalent ligands fibrinogen and vWF facilitates cross-linking of platelets leading to the development of a platelet plug, or aggregate (Nurden & Nurden, 1993). It is therefore logical that agents that can inhibit the binding of ligands to GP IIb/IIIa may inhibit the aggregation of activated platelets.

The results of the platelet aggregation assays show that at a final concentration of 0.5 IU/ml thrombin, the presence of 20 μg/ml of either of the anti-GP IIb/IIIa antibodies RFGP56 or c7E3 was sufficient to prevent aggregation. This is consistent with previous *in vitro* findings (Reverter *et al*, 1996; Pedicord *et al*, 1998) and the clinical benefits of ReoPro™ in the prevention of vessel occlusion following vascular surgical procedures, where the local generation of thrombin and the

exposure of subendothelial proteins may lead to the aggregation of platelets (Califf *et al*, 1995; EPILOG Investigators, 1997; CAPTURE Investigators, 1997).

RFGP37 binds to a vWF binding site on the GPIbα receptor, located in the first leucine rich repeat region near or between residues 35 and 81 (Stephen F Garner, Personal Communication, 1999). Antibodies from both Cymbus and Serotec were able to completely inhibit ristocetin-induced platelet agglutination, confirming that RFGP37 interferes with the binding of vWF to GP Ibα. As discussed in Section 1.2.1.1, there are three vWF binding regions on GP Ibα, one of which is also involved in the binding of thrombin.

The presence of 20 μg/ml of Cymbus RFGP37, as intact antibody or $F(ab')_2$ fragment, against GP Ibα did not inhibit platelet aggregation following stimulation with 0.5 IU/ml of thrombin. However, stimulation of platelets with lower concentrations of thrombin led to increasing inhibition by this antibody, which led to the hypothesis that the role of the GP Ib/IX/V complex as a high affinity thrombin receptor may be significant (De Marco *et al*, 1991). The vWF binding site that includes the epitope for RFGP37 is discrete from the high affinity thrombin binding site, which is located at amino acids 269 - 287 (De Marco *et al*, 1994), but it is possible that steric hindrance or conformational change resulting from the interaction of RFGP37 with GP Ibα may interfere with the interaction of thrombin with its binding site. However, subsequent investigation using fragments of GP Ibα proved this not to be the case, although the use of an ELISA method may have resulted in certain epitopes being obscured so this result cannot be definitive.

The possibility that the inhibitory effects of Cymbus RFGP37, in platelet aggregation and thrombin generation, were artefactual was raised when at 20 μg/ml Serotec RFGP37 failed to inhibit platelet aggregation at any of the concentrations of thrombin tested, despite being able to bind to the GP Ibα fragments. In fact, Serotec RFGP37 appeared to stimulate platelet aggregation, although this was not statistically significant. This indicated different properties of antibodies from the two different manufacturers and also of variable inter-batch properties, and suggested that the inhibitory effect of the original batch of Cymbus RFGP37 (297J) was due to a contaminant that could interfere with the platelet response to weak aggregatory stimuli, such as low concentrations of thrombin. The unavailability of any further supplies of batch 297J made it impossible to identify the differences between the batches.

The intrinsic thrombin generation system described is a platelet-dependent system, demonstrated by the lack of detectable thrombin generation in the absence of platelets. The intrinsic pathway (described in Section 1.1.1) may be supported by the ability of platelets to bind FXII, FXI, FX and HMWK and their provision of negatively charged phospholipids for the interaction of these activated clotting factors (Saito, 1994). The early activation of platelets is indicated by the steep incline and early peak of the control curve in Figure 4.5a.

The presence of anti-GP IIb/IIIa antibodies RFGP56 or c7E3 significantly reduced thrombin generation in the intrinsic system to levels similar to that previously reported in an extrinsic system (Reverter *et al*, 1996), confirming the inhibitory effect of these agents in platelet dependent thrombin generation Cymbus RFGP37

inhibited thrombin generation in the intrinsic system, although to a lesser extent than the anti-GP IIb/IIIa antibodies, and as this was prior to the discovery of the problems with Cymbus RFGP37, this was interpreted as an indication that GP Ibα may also have a stimulatory role in platelet dependent thrombin generation.

Extrinsic thrombin generation using high (630 pM) TF as the stimulus was not inhibited as effectively by the antibodies. Only the strongest inhibitor under intrinsic stimulation, RFGP56, had a significant effect on AUC and peak thrombin concentration. The high level of TF was probably sufficient in itself to drive platelet-independent thrombin generation via interaction with FVII and activation of FX. This theory is reinforced by the high level of thrombin generation seen in the absence of platelets and the absence of any lag time in the control curve in Figure 4.6a. The relatively low inhibition of thrombin generation by all the antibodies may therefore be due to the comparatively minor role being played here by the membrane of activated platelets, as enough FXa may be generated by the high levels of the TF-FVIIa complex to be able to cleave prothrombin in the absence of platelet phospholipid. This is consistent with a previous report that found no inhibition of thrombin generation with c7E3 following stimulation with 200 pM TF (Butenas *et al*, 2001).

When low TF (35 pM) was used to stimulate thrombin generation under static conditions no significant thrombin generation was seen in the absence of platelets, indicating that the thrombin generation here is platelet dependent. This is confirmed by the long lag time before any thrombin is detected in the control curve in Figure 4.7a, which reflects the initiation phase of coagulation before enough

thrombin to activate platelets has been generated. The inhibitory actions of all the antibodies were similar to those seen in the intrinsic system, with the anti-GP IIb/IIIa antibodies RFGP56 and c7E3 reducing the AUC and all antibodies reducing peak thrombin concentration. These results are in agreement with previous reports that showed c7E3 (Reverter *et al*, 1996; Butenas *et al*, 2001) and a specific non-peptide antagonist of GP IIb/IIIa (Herault *et al*, 1998) to affect thrombin generation in a physiologically relevant system similar to that used here. The clinically used concentration of c7E3 is around 3 μg/ml (following a bolus of 0.25 mg/kg) and provides 80 % blockade of GP IIb/IIIa (Centocor product information), therefore the 20 μg/ml of anti-GP IIb/IIIa antibodies used here should be sufficient to provide a similar degree of receptor blockade.

The anti-aggregatory action of anti-GP IIb/IIIa antibodies is one mechanism by which they inhibit platelets. However, anti-GP IIb/IIIa antibodies also interfere with the intracellular processes that follow ligation of GP IIb/IIIa. Isenberg et al (1990) demonstrated that after ligand binding, GP IIb/IIIa is internalised in a cytoskeleton-mediated process. It was also shown that antibodies bound to both GP IIb and GP IIIa are internalised in this manner, but their interference with the cytoskeleton results in inhibition of the formation of filopodia. It is therefore possible that the antibodies used here can also interfere with this process, as engagement of the cytoskeleton precedes and facilitates the formation of filopodia and procoagulant microparticles, formed by pinching off sections of the scrambled platelet membrane (Sims *et al*, 1989).

Neither antibody significantly affected the time of peak thrombin concentration, indicating that the initiation phase of coagulation, prior to platelet activation, is unaffected. The decreased thrombin generation may therefore reflect the expression of a reduced amount of phospholipid that can support fewer tenase and prothrombinase complexes. The exposure of negatively charged phospholipids, as determined by annexin-A5 binding, was indeed reduced by anti-GP IIb/IIIa antibodies to a similar degree as the reduction in thrombin generation. This is in agreement with a previous study that correlated a reduced prothrombinase activity on platelets with reduced exposure of negatively charged phospholipids following treatment with GP IIb/IIIa antagonists (Pedicord *et al*, 1998). The incomplete inhibition of thrombin generation by anti-GP IIb/IIIa antibodies may be the result of stimulation via other pathways such as the PAR-1 thrombin receptor, and may also be due to increased expression of GP IIb/IIIa on the surface of the activated platelet. This is the result of transport from internal stores in the open canalicular system and α-granules, where the inhibitory antibodies may not be able to bind to the receptor, thus facilitating aggregation following the fusion of α-granules with the plasma membrane (Nurden *et al*, 1999).

The presence of HUVEC did not affect the inhibitory action of RFGP56 under static conditions. RFGP56 binds to a conformational epitope across the two subunits of GP IIb/IIIa (Cox, 1991) and is therefore unable to bind to endothelial cells, despite their expression of GP IIIa as part of the vitronectin receptor (integrin $\alpha_V\beta_3$) (Nurden, 1994), and this was confirmed by immunofluoresence. The presence of ECM led to a reduction in the inhibitory capacity of RFGP56, possibly due to platelet activation via other receptors such as the collagen receptors GP Ia/IIa and GP VI,

reducing the contribution of GP IIb/IIIa mediated signalling to the procoagulant activity of the platelet. The introduction of shear stress showed that, independent of the presence of HUVEC or ECM, RFGP56 was most effective under static conditions where the procoagulant activity of platelets follows their activation by thrombin, or arterial conditions where platelet aggregation is critical in order to retain a catalytic surface of activated platelet membranes for the interaction of clotting factor complexes at the site of vascular damage.

Under static conditions neither HUVEC nor ECM had any effect on thrombin generation in the presence of Cymbus RFGP37, which had an apparent, but not statistically significant, inhibitory effect in polystyrene tubes. Reduced effectiveness of RFGP37 may have been expected in the presence of endothelial cells, which express GP Ibα (Wu *et al*, 1997). However, endothelial cells were not seen to bind Serotec RFGP37 by immunofluorescence. The introduction of shear stress led to a dose dependent inhibition of thrombin generation in the presence of both HUVEC and ECM, suggesting that GP Ibα was playing an increased role in platelet activation, possibly by its thrombin receptor function, by its stimulation of platelets via the collagen-vWF pathway, or by its tethering effect under high shear conditions and that RFGP37 was able to inhibit this.

The inhibitory effect of Cymbus RFGP37 in platelet aggregation in response to low concentrations of thrombin indicated that this might be its mode of action in inhibition of thrombin generation. Inhibition of the thrombin receptor function of GP Ibα would reduce the effects of low levels of thrombin in activating platelets, and may therefore act to increase the delay before the peak of the thrombin generation

curve was seen. However, the data were unable to confirm this hypothesis. The inhibition of platelet mediated thrombin generation by an antibody against GP Ibα was contrary to a previous report where the antibody used (6D1) was directed against a different vWF binding region in the leucine rich repeats of the receptor (Reverter *et al*, 1996) from that of RFGP37. Antibody 6D1 has since been shown to inhibit enhanced thrombin generation seen in the presence of fibrin clots (Béguin *et al*, 1999), suggesting an alternative mode of action for this antibody. Detectable levels of soluble fibrin were found in the defibrinated plasma used in the thrombin generation system, and it was therefore possible that the baseline thrombin generation had been potentiated by the presence of soluble fibrin. RFGP37 blocks the binding of vWF to GP Ibα and may therefore interfere with the indirect interaction of fibrin with GP Ibα, thus preventing this potentiation and reducing baseline thrombin generation to normal. However, no difference in the effect of Cymbus RFGP37 was seen when all the soluble fibrin was removed from the defibrinated plasma. In addition, had interference with this pathway been the mode of action of RFGP37, an antibody directed against the GP Ibα binding site on vWF would be expected to show a similar inhibitory effect. However, when the effect of RFFVIII:R/1 on thrombin generation in defibrinated plasma containing soluble fibrin was tested, no inhibition was seen. This indicates that the fibrin-vWF-GP Ibα pathway is not the site of Cymbus RFGP37's inhibitory effect. Previous reports have confirmed the role of GP Ibα in the tethering of platelets to areas of vascular damage under conditions of high shear stress, as described in Section 1.2.1.1. If Cymbus RFGP37 was able to interfere with this function of GP Ibα then it may inhibit thrombin generation by preventing the accumulation of platelets with negatively-charged phospholipids exposed on their membranes at the vessel wall. No

significant inhibitory effects were seen with Serotec RFGP37 under any of the conditions tested, lending more weight to the hypothesis that the inhibitory effects of Cymbus RFGP37 had been artefactual and that although RFGP37 is able to inhibit vWF binding to GP Ibα, it does not have a sufficient inhibitory effect on platelet-mediated thrombin generation.

As both RFGP56 and Cymbus RFGP37 were exhibiting inhibitory effects on thrombin generation, and are directed against different platelet membrane receptors, studies where the antibodies were used in combination were performed to determine if an additive or synergistic effect could be seen. In the presence of HUVEC, no differences were seen between the effects of RFGP56 alone and in combination with RFGP37, from either source, at any shear rate tested. However, in the presence of ECM, an additive effect of RFGP56 and Cymbus RFGP37 was seen under low venous shear conditions (18 s^{-1}), especially at the highest concentration tested (80 μg/ml). This result may have indicated a role for both receptors in the procoagulant activity of platelets under flow conditions in areas of vascular damage and led to the initiation of the further studies at higher shear rates. Further investigations using Serotec RFGP37 were unable to reproduce the original data and further work was performed to characterise the Serotec antibody. These experiments showed that Serotec RFGP37 was able to bind to GP Ibα and inhibit its vWF binding function, but not its function as a thrombin receptor.

The studies on the exposure of negatively charged phospholipids on the platelet membrane revealed more information about the mode of action of anti-GP IIb/IIIa antibodies, which reduced the number of platelets that had undergone membrane

scrambling and therefore limited the assembly of phospholipid-dependent enzymatic complexes. Stimulatory effects of anti-GP Ibα antibodies have been noted previously (Deckmyn *et al*, 1997; Yanabu *et al*, 1997; Cauwenberghs *et al*, 2001), but the apparent Fc-independent stimulatory effect of Cymbus RFGP37 was paradoxical to the antibody's effect in the thrombin generation tests, where it was inhibitory under certain conditions, although a non-significant increase in platelet aggregation was noted with Serotec RFGP37 following thrombin stimulation, and also indicated by its aggregatory effect on platelets studied using immunofluoresence. The possibility of a platelet-independent effect of Cymbus RFGP37 on thrombin generation was considered, but no statistically significant inhibition was seen in thrombin generation tests performed with platelet poor plasma. There is no clear explanation for these results, and without further supplies of the suspect batch of antibody it is impossible to test any hypotheses. The most likely explanation is the presence of a soluble contaminant, but a candidate that activates platelets yet inhibits coagulation is not obvious. The results of the Serotec RFGP37 confirm that the original choice of this antibody as a negative control for RFGP56 in the thrombin generation experiments was appropriate, and had the early results been viewed more sceptically further analysis may have revealed the reason for the unusual behaviour of Cymbus RFGP37.

4.5 Summary

A platelet-dependent thrombin generation system was established, and the inhibitory effects of antibodies against GP IIb/IIIa (RFGP56 and c7E3) and GP Ibα (RFGP37) were investigated. RFGP37 manufactured by Cymbus showed inhibitory activity that platelet aggregation studies suggested was due to inhibition of the thrombin

receptor function of GP Ibα. However, RFGP37 made by Serotec showed no inhibitory effect on thrombin generation and was unable to inhibit thrombin binding to GP Ibα, leading to the conclusion that the inhibitory effects of Cymbus RFGP37 were the result of a soluble contaminant, and that inhibition of vWF binding to GP Ibα is not sufficient to inhibit the contribution of platelets to coagulation. RFGP56 was able to inhibit thrombin generation, and was most effective under either static or arterial flow conditions and in the presence of HUVEC rather than ECM. Only partial inhibition of thrombin generation was possible, suggesting that platelets may also become activated via other pathways, such as ligation of collagen receptors. In addition to the anti-aggregatory activity of RFGP56, its mode of action in inhibiting coagulation was shown to be a reduction of the exposure of negatively charged phospholipids that serve as templates for the assembly of complexes of coagulation enzymes on the outer membrane of platelets.

CHAPTER 5

HEPARIN & HIRUDIN

5.1 Introduction

Unfractionated and low molecular weight heparins are widely used antithrombotic drugs, and small-molecule thrombin inhibitors such as hirudin are becoming established as alternatives to heparin. The relative benefits of these different agents in different clinical indications may be due to pharmacokinetic or physiochemical differences but may also be due to the different specificities of these agents against individual proteases of the coagulation cascade.

5.1.1 The relative contribution of anti-Xa and anti-IIa activity

A number of experimental studies have investigated the contribution of anti-Xa and anti-IIa activities to the inhibition of coagulation. The results do not all agree, and vary depending on the procoagulant stimulus, the flow conditions, the reactive surface and whether the model is *in vivo* or *in vitro* (Buchanan *et al*, 1985; Amar *et al*, 1990; Vlasuk *et al*, 1991; Orvim *et al*, 1995; Diquelou *et al*, 1995b; Bossavy *et al*, 1998; Bossavy *et al*, 1999; Shimbo *et al*, 2002). Standardised studies with physiologically relevant flow conditions, procoagulant stimuli and reactive surfaces would therefore be useful in determining the relative contributions of anti-Xa and anti-IIa activity.

Unfractionated heparin, low molecular weight heparin and hirudin are useful tools for this study as they have varied inhibitory activities against FXa and thrombin, but it is difficult to make direct potency comparisons between them. Comparisons on a molar basis are not possible as heparin is a heterogeneous mixture of different length polysaccharide chains. Comparison on a functional basis is also difficult as heparin

acts indirectly as a catalyst for antithrombin, whereas hirudin inhibits thrombin directly. In order to avoid confusion between the catalytic activity of heparin, usually referred to as anti-IIa activity, and the direct inhibition of thrombin by hirudin, the term 'thrombin inhibitory activity' will be used to refer to the activities of both heparin and hirudin. The difference in the mode of action leads to non-parallelism when dilution series of heparin and hirudin are tested in coagulation-based assays such as the activated partial thromboplastin time (APTT), making comparative potency analysis invalid (European Pharmacopoeia, 2002). The APTT is routinely used as a laboratory test for the monitoring of patients receiving antithrombotic therapy, and the therapeutic range is considered to be a 1.5 – 2.5 fold increase of the control clotting time (Hirsh *et al*, 2001). The concentration (in μg/ml) of each inhibitor required to double the control APTT (a ratio of 2.0) was therefore determined in order to allow a single-point comparison between the compounds and to provide a reference point for comparison with clinical data.

Platelet factor 4 (PF4) is a 70 amino acid protein (MW 7 800) that is stored in the α-granules of platelets. It is released when platelets become activated and circulates as a tetramer or bound to a proteoglycan carrier. It also binds to heparin, neutralising its activity, and binds with lower affinity to heparin-like molecules on the endothelium from where it is released following heparin therapy (Niewiarowski, 1994). It is possible that the activation of platelets in these studies may lead to the release of PF4 from platelets and the presence of heparin may release PF4 from HUVEC, resulting in the neutralisation of some heparin activity and thereby influencing the results. An investigation into the concentration of PF4 in post-shear platelet plasma was therefore undertaken.

Studies were designed to investigate how the anti-Xa and thrombin-inhibitory activities of the different compounds contribute to their antithrombotic effects under different physiological conditions. Consideration was given to the differences in the modes of action of the inhibitors, and the influence of shear stress on the process of coagulation.

5.2 Materials and methods

5.2.1 Inhibitors

Unfractionated heparin was the 5th International Standard (97/578, NIBSC, UK) with a specific activity of 230 IU/mg (for both anti-Xa and anti-IIa). Low molecular weight heparin was Bemiparin (Rovi, Spain) with a specific activity of 100 anti-Xa IU/mg and 10 anti-IIa IU/mg. Hirudin was Refludan (Hoechst Marion Roussel, Frankfurt am Main, Germany), a recombinant desulfato hirudin with a specific activity of 20 000 anti-thrombin units (ATU)/mg (Colin Longstaff, Personal Communication, 2002). For further information on potency assays of hirudin see Longstaff *et al* (1993).

5.2.2 Activated partial thromboplastin time (APTT)

The APTT test was performed on Amelung KC4A coagulometers (Brownes, Reading, UK) using APTT reagent from Dade Behring (Marburg, Germany) consisting of purified soy phosphatides in 10^{-4} M ellagic acid (Dade® Actin® FS Activated PTT Reagent) and 25 mM $CaCl_2$. Dilutions of the inhibitors were prepared in TBS and 100 µl of each dilution was added to a cuvette. This dilution was mixed with 100 µl of normal pooled plasma (prepared from a minimum of 4 donors; North London Blood Transfusion Centre, Colindale, UK), and 100 µl of APTT reagent was then added. This mixture was incubated at 37 °C for 5 minutes before the addition of 100 µl of $CaCl_2$. The clotting times were recorded and dose response curves prepared.

5.2.3 Thrombin generation tests under static and flow conditions

The inhibitory effects of UFH, LMWH and hirudin on thrombin generation were studied under static and flow conditions in the presence of HUVEC or ECM using low TF (35 pM) as the stimulus as described in Section 2.8.

5.2.4 Platelet Factor 4 assay

The Hyphen Biomed Zymutest PF4 kit (Quadratech, Epsom, UK) was used to quantify the amount of PF4 that was released by platelets during the course of a perfusion experiment. This is a sandwich ELISA kit that uses rabbit anti-human PF4 polyclonal antibodies to capture and detect PF4 in diluted samples of plasma. The recommended anticoagulants for the collection of blood were ETP (EDTA, theophylline and prostaglandin E_1) or CTAD (citrate, theophylline, adenosine and dipyridamole), which are designed to inhibit platelet activation, and therefore any further release of PF4. In order to adapt this method for use on samples of plasma that had been exposed to shear stress in the flow system, an isotonic collection buffer that included these platelet inhibitors was required. A modified citrate saline (150 mM NaCl, 109 mM trisodium citrate; reagents from BDH, Poole, UK) was prepared containing 15 mM theophylline, 3.7 mM adenosine and 0.17 mM dipyridamole (all from Sigma, Poole, UK), with the concentrations of the inhibitors based on advice received from the suppliers of the kit and from a manufacturer of blood collection tubes (Sarstedt, Leicester, UK).

Platelet plasma was prepared as usual and either stimulated with TF/Ca^{2+} or Ca^{2+} alone and then passed through the flow chamber, which contained a HUVEC-coated coverslip. A control where the TF/Ca^{2+} stimulated plasma was transferred to a

culture well containing a HUVEC-coated coverslip was also included. Subsamples of 20 µl were collected at 60 second intervals for 32 minutes and then centrifuged (1 000 g for 5 minutes) to remove any platelets from the supernatant, which was then aspirated and assayed for PF4 content.

5.3 Results

5.3.1 APTT

Dose response curves were prepared on a weight basis in order to compare the inhibitory effects of UFH, LMWH and hirudin, and are shown in Figure 5.1. The clotting time of the normal plasma in the absence of any inhibitors was 38.3 ± 0.68 seconds (n = 6). Lines were fitted to the data points with Microsoft Excel and the equation of each fitted line was used to calculate the dose required to double the APTT to 76.5 seconds (an APTT ratio of 2.0). The concentrations (with 95 % confidence intervals) required to achieve this effect were 1.03 (1.01 – 1.06), 16.49 (15.64 – 17.36) and 0.56 (0.47 – 0.67) μg/ml for UFH, LMWH and hirudin respectively.

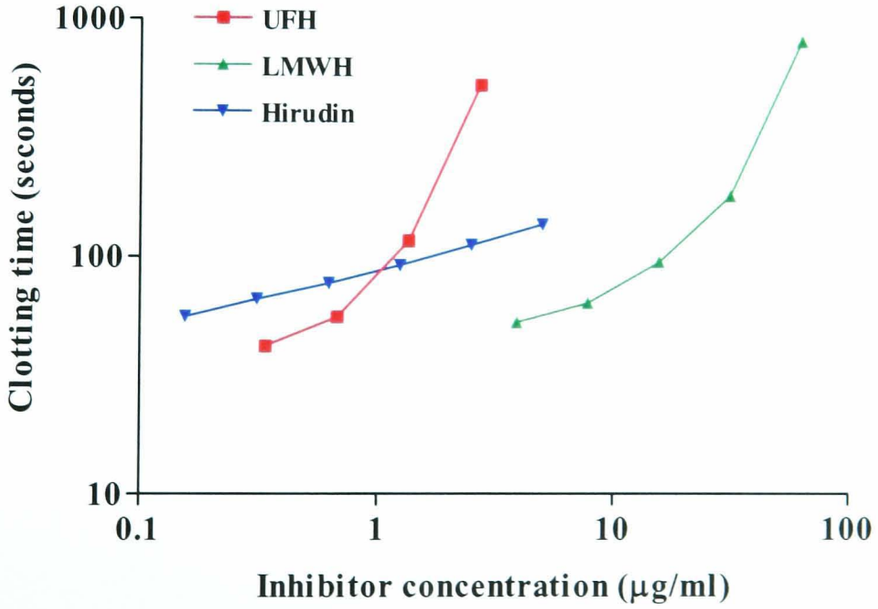


Figure 5.1 *The inhibitory effects of UFH, LMWH and hirudin in the APTT assay.*

Dilutions of the inhibitors were prepared in TBS and 100 μl of the dilution incubated with 100 μl of normal pooled plasma and 100 μl of Dade® Actin® FS APTT reagent at 37 °C for 5 minutes. 100 μl of $CaCl_c$ was added and the clotting time recorded on Amelung KC4A coagulometers. Each point is the mean of duplicate determinations; the error bars for standard error of the mean (SEM) are smaller than the data points.

5.3.2 Thrombin generation under static and flow conditions

Examples of thrombin generation curves from single experiments are shown in Figure 5.2 to illustrate the effect of the inhibitors under static, venous and arterial shear conditions in the presence of ECM. Figure 5.2a shows that under static conditions hirudin delays the peak of the curve but has little effect on the shape or height of the curve at concentrations up to 0.5 μg/ml. In contrast, Figure 5.2b shows the effect of LMWH under venous conditions to be reduction of the height of the curves as well as delaying them. UFH was less effective in the reduction of peak height under these conditions (not shown, see Figures 5.3a, 5.7a, & 5.10a). Under arterial conditions, UFH shows both reduction and delay of the peak (Figure 5.2c), with a delayed peak being followed by reduced peaks as the inhibitor concentration increased. LMWH was more effective in the reduction of peak height and delay of the peak (not shown, see Figures 5.3b, 5.7b & 5.10b).

Fig 5.2a ECM + Hirudin @ 0 s^{-1}

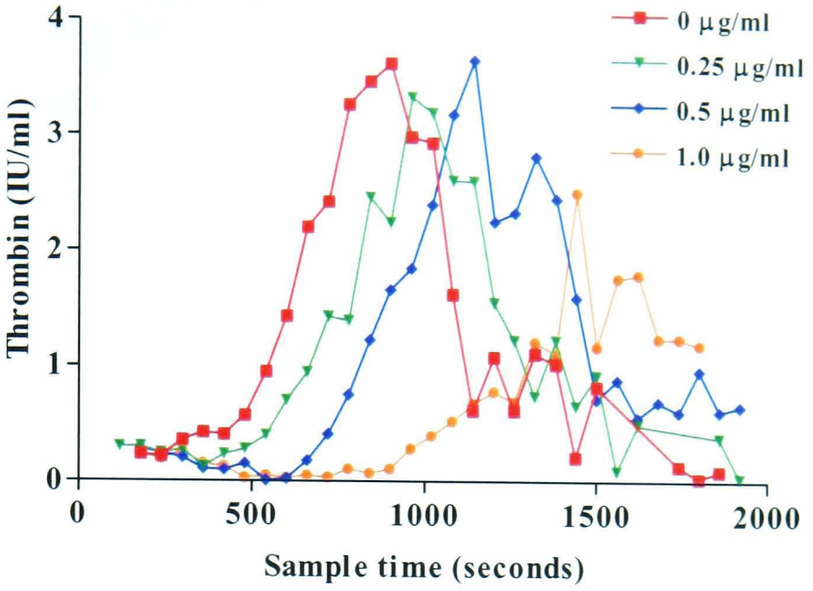


Fig 5.2b ECM + LMWH @ 178 s^{-1}

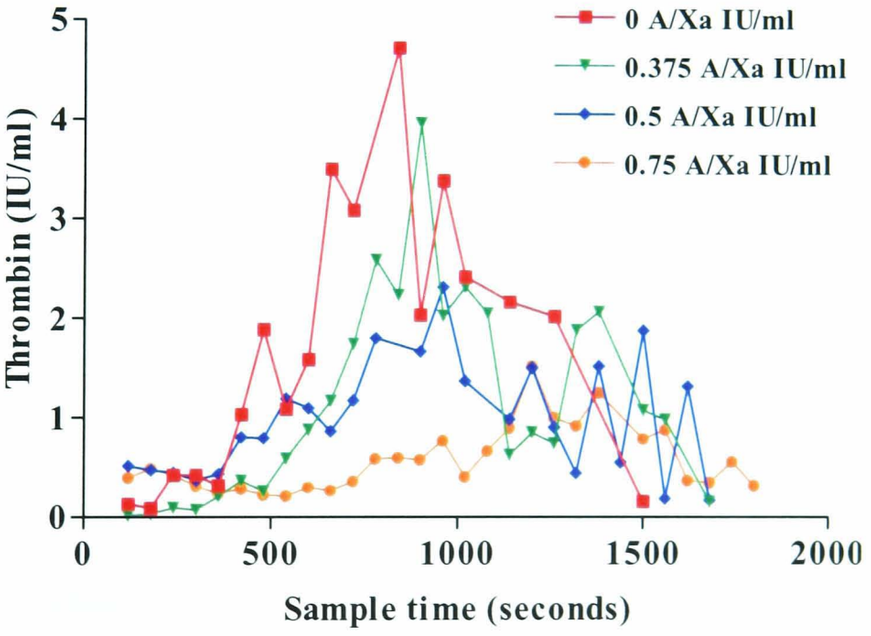


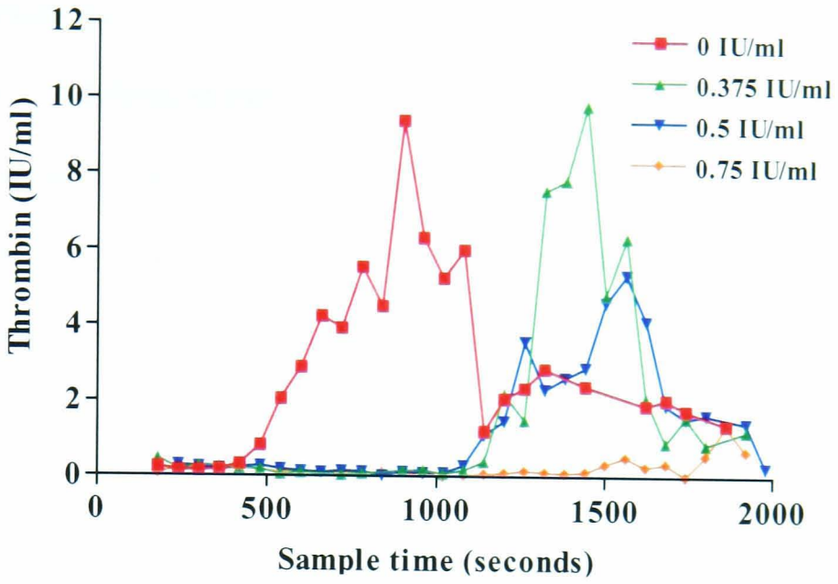
Figure 5.2 *Representative thrombin generation curves from single experiments in the presence of ECM.*

800 µl of defibrinated plasma containing 300 x 10^6 platelets was incubated for 30 minutes at 37 °C with 25 µl of TBS containing the inhibitor under test. Thrombin generation was initiated with 175 µl of 100 mM $CaCl_2$ containing recombinant tissue factor to give a final concentration of 35 pM. Timed subsamples were taken into EDTA stop buffer for determination of thrombin concentration with chromogenic substrate S2238.

a) static conditions and varying concentrations of hirudin;

b) venous shear and LMWH;

Fig 5.2c ECM + UFH @ 600 s^{-1}



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c) arterial shear and UFH.

Data from individual experiments were combined by calculating the area under the curve (AUC), the peak height and the time of the peak as a percentage of that seen in the absence of any inhibitor. This allowed data from separate experiments to be combined in a manner that controlled for variability in the platelets from individual donors. Dose response curves for each parameter were then compiled for each inhibitor in the presence of either HUVEC or ECM and under each shear condition (static, venous or arterial). These curves are presented in Figures 5.3 to 5.13, showing arithmetic mean ± standard deviation from at least 3 experiments. Curves were fitted to the data points as described in Section 2.12 in order to calculate the IC_{50} for each inhibitor under each set of conditions and these are presented for the AUC and peak height in Tables 5.1 and 5.2, respectively.

5.3.2.1 *Effect of inhibitors on total thrombin generation (AUC) under static conditions*

Under static conditions, significantly more of each inhibitor was required to inhibit thrombin generation to 50% of control levels (IC_{50}) over ECM than over HUVEC ($p < 0.001$ in all cases). The IC_{50} data were determined from weighted regression of logit response on log dose and are shown in Table 5.1, with the LMWH data calculated on the basis of anti-Xa potency. These results are consistent with the observation that under static conditions ECM is more thrombogenic than HUVEC and therefore a greater quantity of inhibitor is required to reduce thrombin generation by 50%.

Table 5.1 *IC_{50} data (with 95% confidence limits) for total thrombin generation (AUC).*

	HUVEC			ECM		
Shear rate (s^{-1})	UFH (IU/ml)	LMWH (A/Xa IU/ml)	Hirudin (μg/ml)	UFH (IU/ml)	LMWH (A/Xa IU/ml)	Hirudin (μg/ml)
0	0.18 (0.05 – 0.70)	0.27 (0.14 – 0.51)	0.98 (0.62 – 1.52)	0.34 (0.25 – 0.46)	0.57 (0.39 – 0.85)	2.79 (1.12 – 6.98)
178	0.40 (0.28 – 0.55)	0.68 (0.31 – 1.58)	>4.00†	0.41 (0.18 – 0.76)	0.44 (0.18 – 1.04)	>4.00†
600	0.54 (0.41 – 0.72)	0.53 (0.41 – 0.69)	>4.00†	0.47 (0.28 – 0.78)	0.59 (0.31 – 1.12)	>4.00†

† *The 50% response is outside the range of observed responses.*

5.3.2.2 *Effect of inhibitors on total thrombin generation (AUC) under flow conditions*

As summarised in Table 5.1, a higher concentration of each inhibitor was required for inhibition of total thrombin generation under flow conditions compared to static conditions. Figures 5.3a and 5.4a show the dose response curves for UFH and LMWH in the presence of HUVEC. Statistical analysis of these dose response curves showed a significant shift to the right under both venous (178 s^{-1}) and arterial (600 s^{-1}) shear conditions ($p < 0.001$). In the presence of ECM the shift in the UFH and LMWH dose response curves is less significant ($p = 0.053$) and confirms the relatively procoagulant condition of the ECM independent of the shear rate (Figures 5.3b and 5.4b).

Hirudin was less effective in reducing total thrombin generation than UFH and LMWH under flow conditions. After the initial 40 % reduction in thrombin generation with concentrations of hirudin up to 1 μg/ml, no further decrease was observed with increased concentrations of hirudin. Consequently, it was not possible to derive valid IC_{50} values as the 50 % response was outside the range of observed responses (Figure 5.5).

Fig 5.3a HUVEC

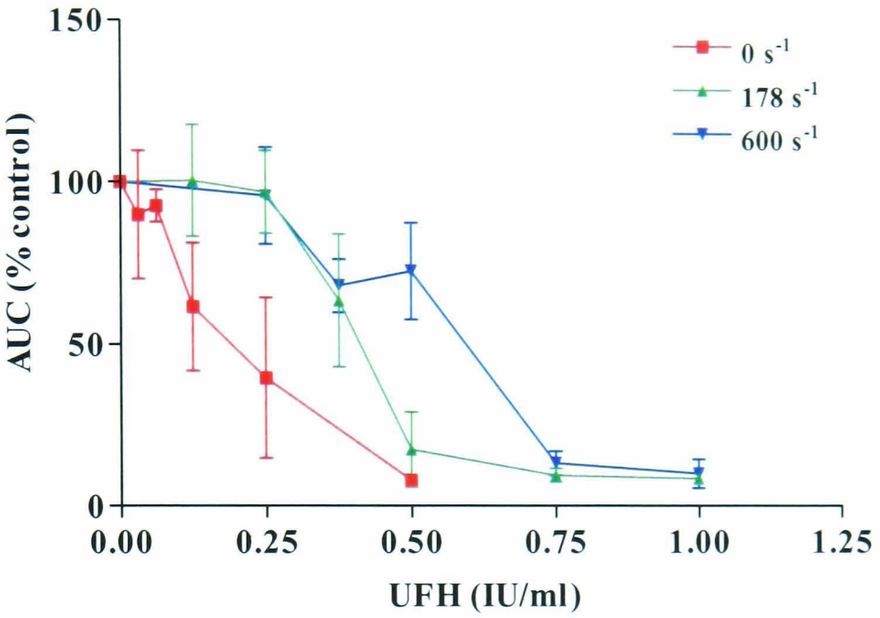

Fig 5.3b ECM

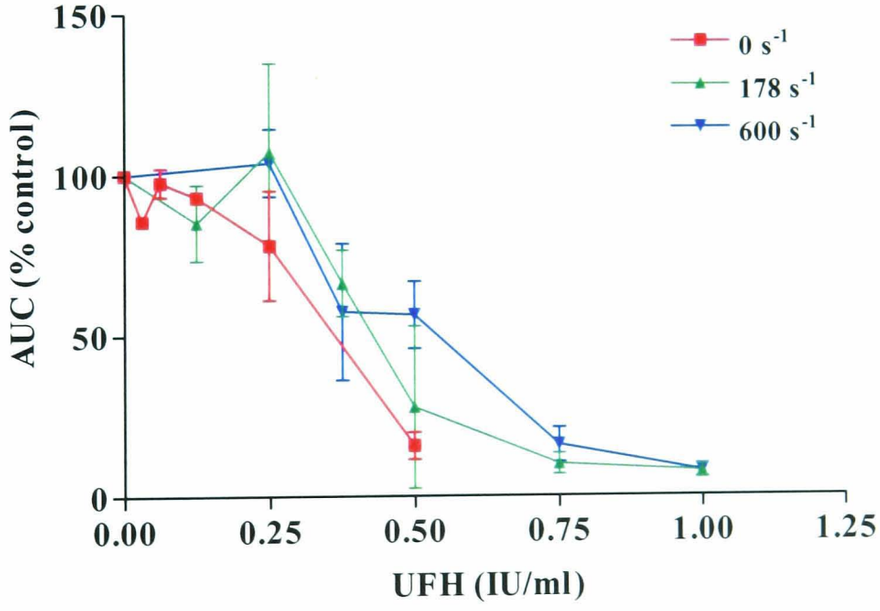

Figure 5.3 *Dose response curves for inhibition of total thrombin generation (AUC) by UFH.*

800 µl of defibrinated plasma containing 300 x 10^6 platelets was incubated for 30 minutes at 37 °C with 25 µl of TBS containing UFH. Thrombin generation was initiated in the presence of a) HUVEC or b) ECM. with 175 µl of 100 mM $CaCl_2$ containing recombinant tissue factor to give a final concentration of 35 pM. Timed subsamples were collected into EDTA stop buffer for determination of thrombin concentration with chromogenic substrate S2238.

Fig 5.4a HUVEC

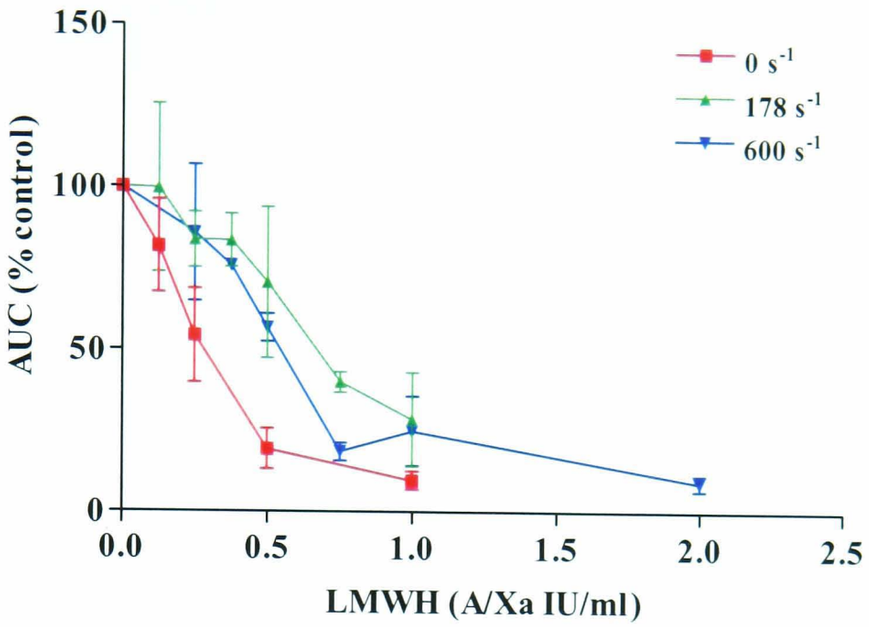

Fig 5.4b ECM

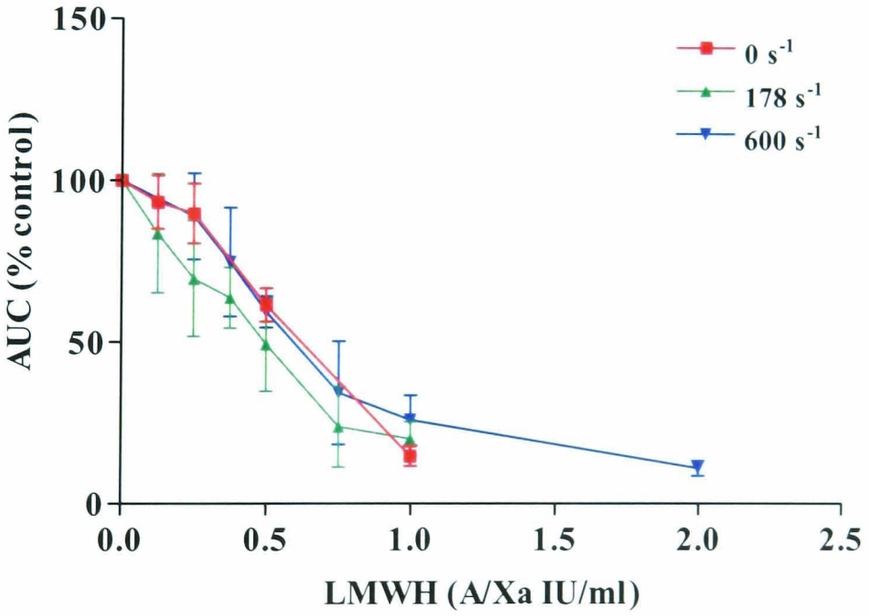

Figure 5.4 *Dose response curves for inhibition of total thrombin generation (AUC) by LMWH.*

800 µl of defibrinated plasma containing 300 x 10^6 platelets was incubated for 30 minutes at 37 ºC with 25 µl of TBS containing LMWH. Thrombin generation was initiated in the presence of a) HUVEC or b) ECM. with 175 µl of 100 mM $CaCl_2$ containing recombinant tissue factor to give a final concentration of 35 pM. Timed subsamples were collected into EDTA stop buffer for determination of thrombin concentration with chromogenic substrate S2238.

Fig 5.5a HUVEC

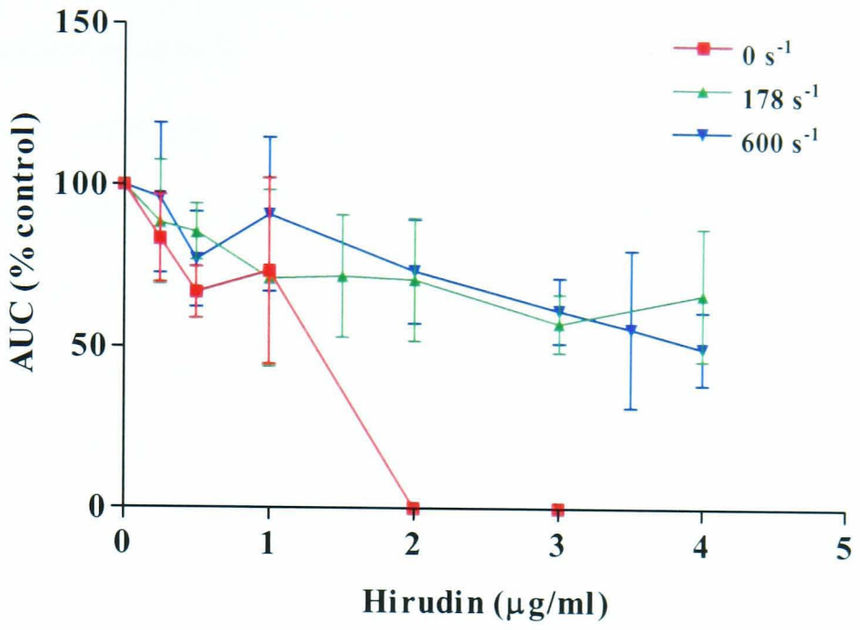

Fig 5.5b ECM

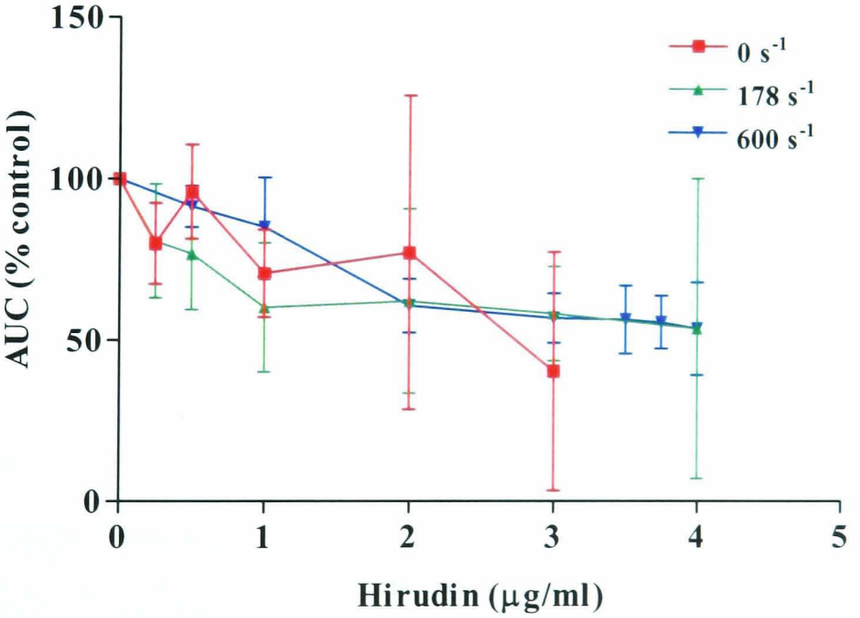

Figure 5.5 *Dose response curves for inhibition of total thrombin generation (AUC) by hirudin.*

800 µl of defibrinated plasma containing 300 x 10^6 platelets was incubated for 30 minutes at 37 °C with 25 µl of TBS containing hirudin. Thrombin generation was initiated in the presence of a) HUVEC or b) ECM. with 175 µl of 100 mM $CaCl_2$ containing recombinant tissue factor to give a final concentration of 35 pM. Timed subsamples were collected into EDTA stop buffer for determination of thrombin concentration with chromogenic substrate S2238.

5.3.2.3 *Comparison of UFH, LMWH and hirudin on the basis of thrombin-inhibitory activity*

Under static conditions and compared on an anti-Xa unit basis, the IC_{50} is significantly higher for LMWH than for UFH ($p < 0.001$), indicating the efficacy of thrombin-inhibitory activity. A comparison on the basis of thrombin-inhibitory units in Figure 5.6 shows that the IC_{50} for LMWH is significantly lower than for UFH ($p < 0.001$). The reason for this difference is likely to be the anti-Xa activity of LMWH (which is 10-fold higher than its thrombin-inhibitory activity) contributing to the inhibitory effect. This indicates that under static conditions both thrombin-inhibitory activity and anti-Xa activity are effective in the inhibition of thrombin generation.

Comparisons of heparin with hirudin are difficult for reasons discussed previously, but Figure 5.6 shows the dose response curves for hirudin expressed in ATU, alongside UFH and LMWH on the basis of anti-IIa IU. Although these units are not comparable this illustrates that high concentrations of hirudin can, like heparin, completely inhibit thrombin generation under static conditions, confirming the efficacy of thrombin-inhibitory activity.

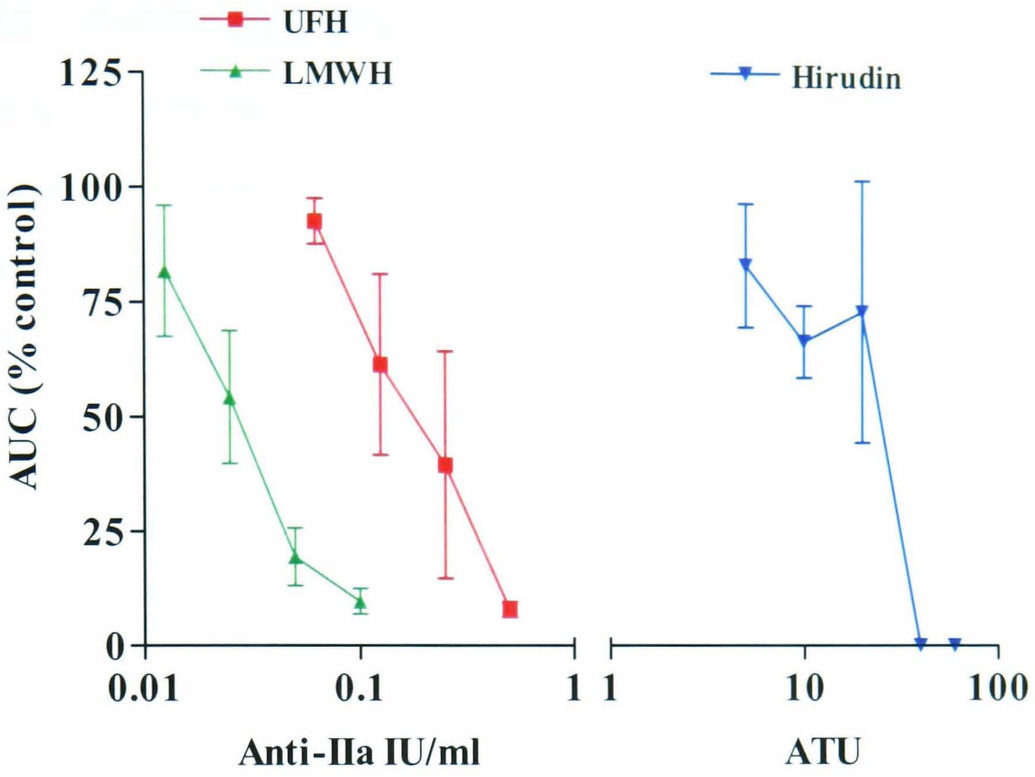

Figure 5.6 *Dose response curves for the inhibition of total thrombin generation under static conditions by UFH, LMWH and hirudin.*

Data are plotted on the x-axis according to anti-IIa international units (IU) for UFH & LMWH or anti-thrombin units (ATU) for hirudin. These units are not comparable, but give an indication of the efficacy of the different inhibitors.

5.3.3 Peak thrombin generation

The height of the thrombin generation curve at its peak was measured in order to characterise the nature of any inhibition seen, and the IC_{50} data are shown in Table 5.2. These data are quite disperse with some non-linear responses and it was difficult to find significant differences between the treatments. However, a trend was seen for a decrease in the peak height as inhibitor concentration increased (following the pattern seen with AUC). No significant differences were seen within the UFH data (Figure 5.7). With LMWH the only significant difference was a higher peak at 178 s^{-1} over HUVEC ($p < 0.01$, Figure 5.8). Analysis of variance (accounting for shear rate and HUVEC/ECM differences) revealed UFH was less effective in reducing the peak height than LMWH ($p < 0.05$).

Hirudin significantly reduced the peak height in static conditions over HUVEC when compared to all other shear conditions and all ECM results ($p < 0.01$, Figure 5.9). This is in keeping with the observation that the least amount of thrombin is generated in static conditions in the presence of HUVEC (see Section 3.3.3.4).

Table 5.2 *IC_{50} data (with 95% confidence limits) for peak height*

	HUVEC			ECM		
Shear rate (s^{-1})	UFH (IU/ml)	LMWH (A/Xa IU/ml)	Hirudin (µg/ml)	UFH (IU/ml)	LMWH (A/Xa IU/ml)	Hirudin (µg/ml)
0	0.19 (0.08 – 0.29)	0.27 (0.06 – 1.47)	1.17 (0.70 – 1.87)	0.41 (0.05 – 0.55)	0.47†† (0.33 – 0.66)	2.34 (1.64 – 3.74)
178	0.39†† (0.16 – 0.54)	0.62 (0.09 – 3.11)	>4.00†	0.43 (0.23 – 0.69)	0.49 (0.20 – 0.93)	3.08 (2.31 – 4.31)
600	0.61 (0.24 – 0.91)	0.51 (0.03 – 8.64)	>4.00†	0.59 (0.29 – 1.30)	0.62 (0.15 – 2.58)	3.67 (3.05 – 4.42)

† *Estimated values only as the 50% response is outside the range of observed responses.*
†† *Analysis was statistically non-linear*

Fig 5.7a HUVEC

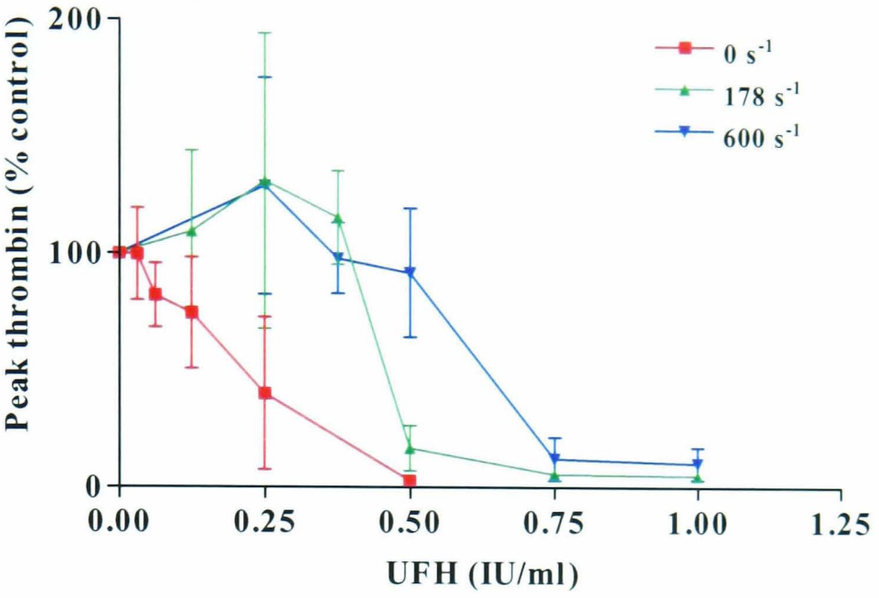

Fig 5.7b ECM

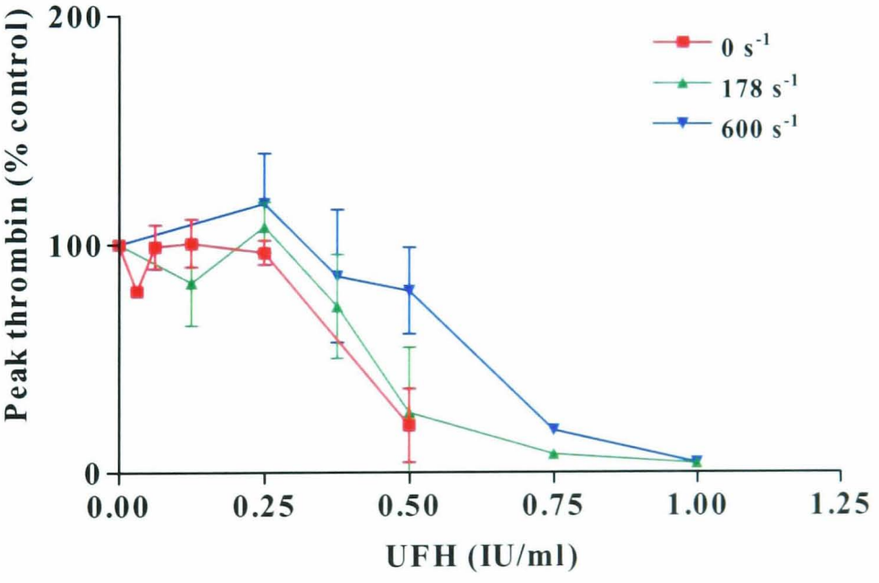

Figure 5.7 *Dose response curves for inhibition of peak thrombin generation by UFH.*

800 µl of defibrinated plasma containing 300 x 10^6 platelets was incubated for 30 minutes at 37 °C with 25 µl of TBS containing UFH. Thrombin generation was initiated in the presence of a) HUVEC or b) ECM. with 175 µl of 100 mM $CaCl_2$ containing recombinant tissue factor to give a final concentration of 35 pM. Timed subsamples were collected into EDTA stop buffer for determination of thrombin concentration with chromogenic substrate S2238.

Fig 5.8a HUVEC

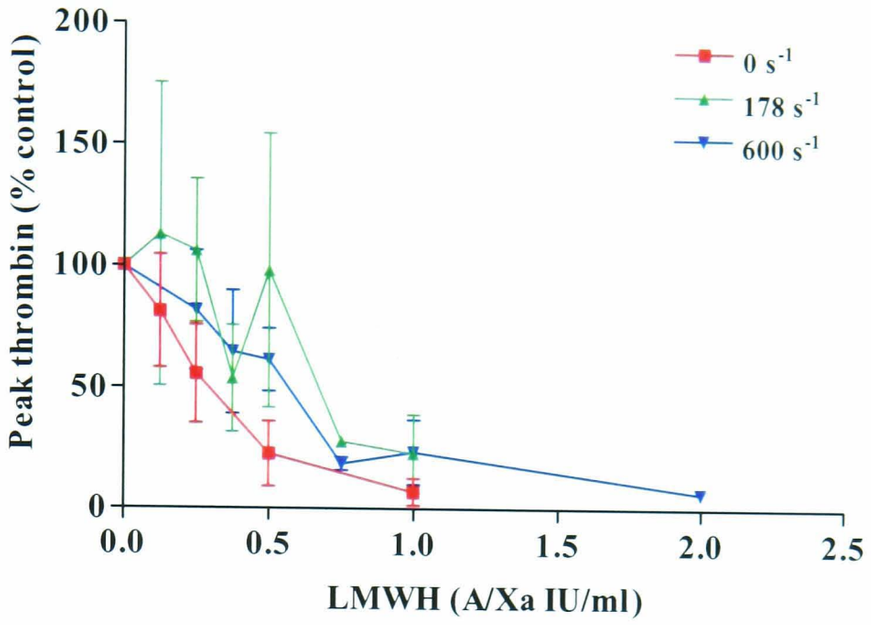

Fig 5.8b ECM

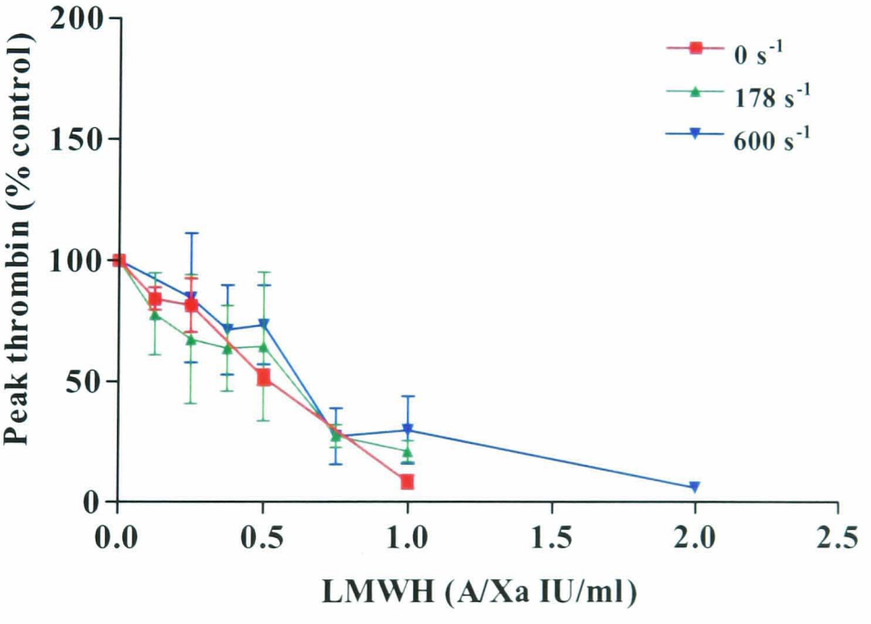

Figure 5.8 *Dose response curves for inhibition of peak thrombin generation by LMWH.*

800 µl of defibrinated plasma containing 300 x 10^6 platelets was incubated for 30 minutes at 37 °C with 25 µl of TBS containing LMWH. Thrombin generation was initiated in the presence of a) HUVEC or b) ECM. with 175 µl of 100 mM $CaCl_2$ containing recombinant tissue factor to give a final concentration of 35 pM. Timed subsamples were collected into EDTA stop buffer for determination of thrombin concentration with chromogenic substrate S2238.

Fig 5.9a HUVEC

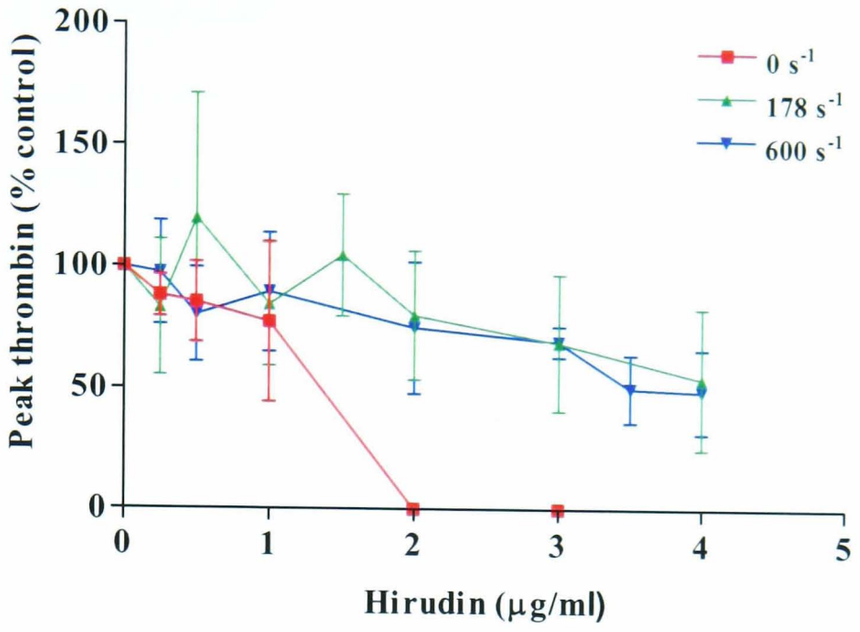

Fig 5.9b ECM

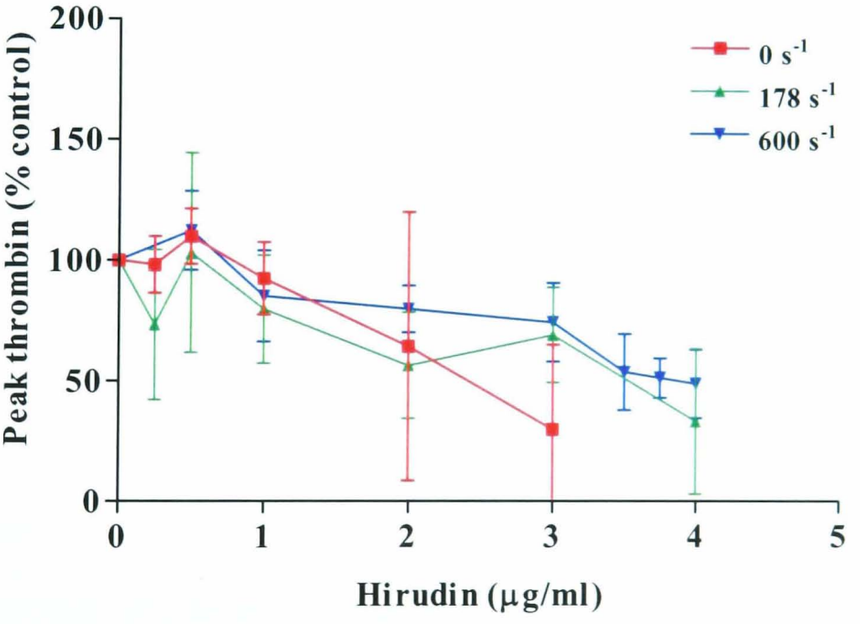

Figure 5.9 *Dose response curves for inhibition of peak thrombin generation by hirudin.*

800 μl of defibrinated plasma containing 300 x 10^6 platelets was incubated for 30 minutes at 37 °C with 25 μl of TBS containing hirudin. Thrombin generation was initiated in the presence of a) HUVEC or b) ECM. with 175 μl of 100 mM $CaCl_2$ containing recombinant tissue factor to give a final concentration of 35 pM. Timed subsamples were collected into EDTA stop buffer for determination of thrombin concentration with chromogenic substrate S2238.

5.3.4 Time of peak thrombin generation

The time at which the peak of the thrombin generation curve was reached was also studied. In some, but not all, experiments with high concentrations of inhibitors no thrombin generation at all was seen during the subsampling period (even when this was extended to 64 minutes) and this variation made it difficult to assign statistical significance to any differences seen. However, there was a trend for the thrombin peak to occur later in the presence of any of the inhibitors.

The different shear rates did not affect the delay before the peak was seen in the presence of either UFH or LMWH (Figures 5.10 and 5.11). However, with hirudin the introduction of shear stress caused the peak to appear earlier than in static conditions. This was significant with hirudin at concentrations between 2 and 4 μg/ml in the presence of HUVEC, when the peak time under static conditions was earlier than at 178 or 600 s^{-1} (both $p < 0.01$; Figure 5.12). Over ECM, the peak time was significantly earlier at 600 s^{-1} than in static conditions for concentrations of hirudin between 1 and 4 μg/ml ($p < 0.01$).

Representative thrombin generation curves using the dose approximately equivalent to the static AUC IC_{50} for hirudin (see Table 5.1) over HUVEC (1 μg/ml) and ECM (3 μg/ml) are shown in Figure 5.13 to illustrate the delay in the appearance of the thrombin generation peak.

Fig 5.10a HUVEC

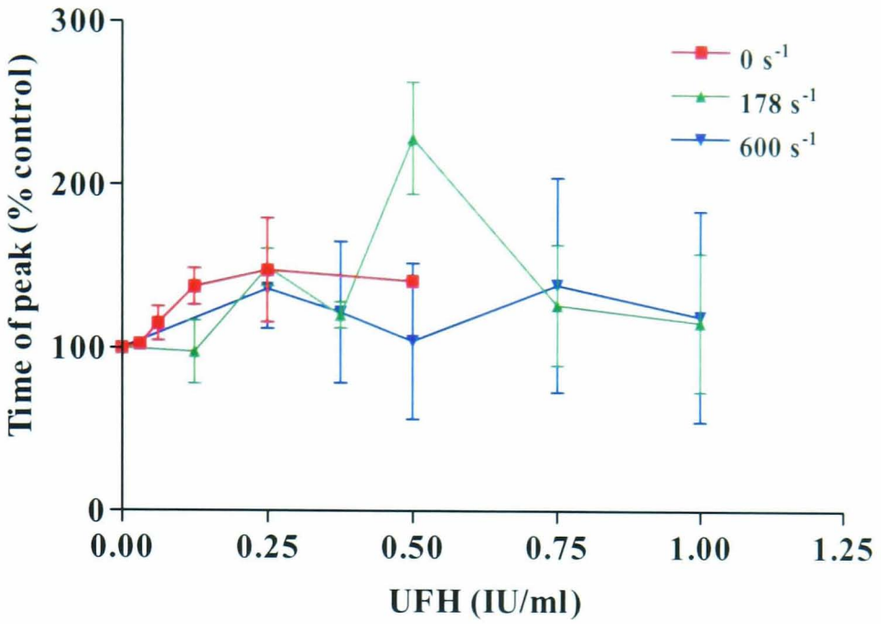

Fig 5.10b ECM

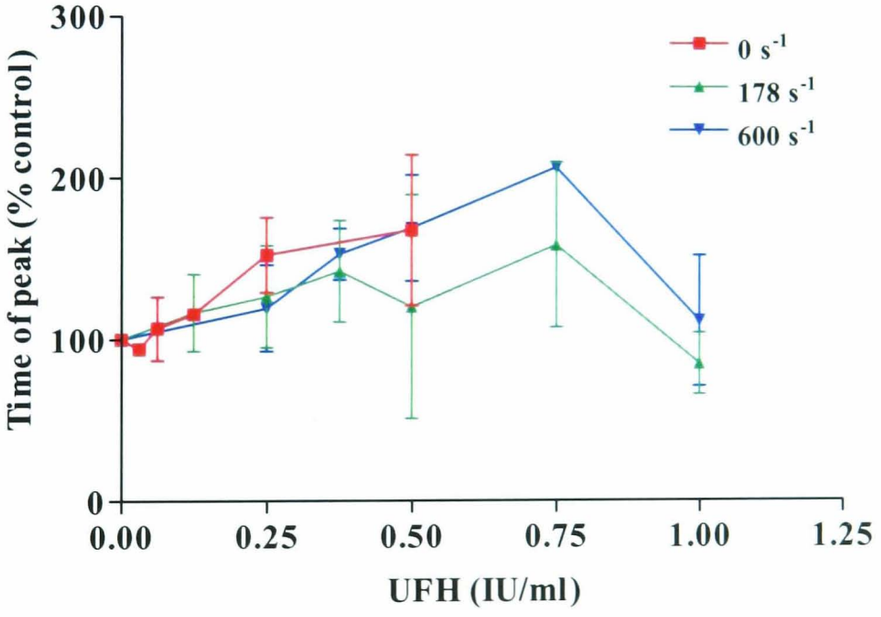

Figure 5.10 *Dose response curves for time of peak thrombin with UFH.*

800 µl of defibrinated plasma containing 300 x 10^6 platelets was incubated for 30 minutes at 37 °C with 25 µl of TBS containing UFH. Thrombin generation was initiated in the presence of a) HUVEC or b) ECM. with 175 µl of 100 mM $CaCl_2$ containing recombinant tissue factor to give a final concentration of 35 pM. Timed subsamples were collected into EDTA stop buffer for determination of thrombin concentration with chromogenic substrate S2238.

Fig 5.11a HUVEC

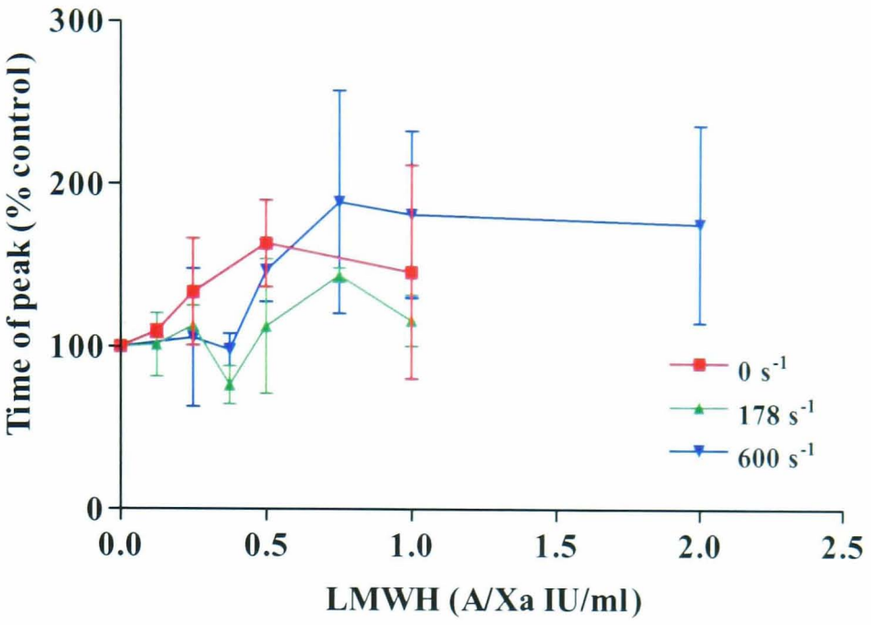

Fig 5.11b ECM

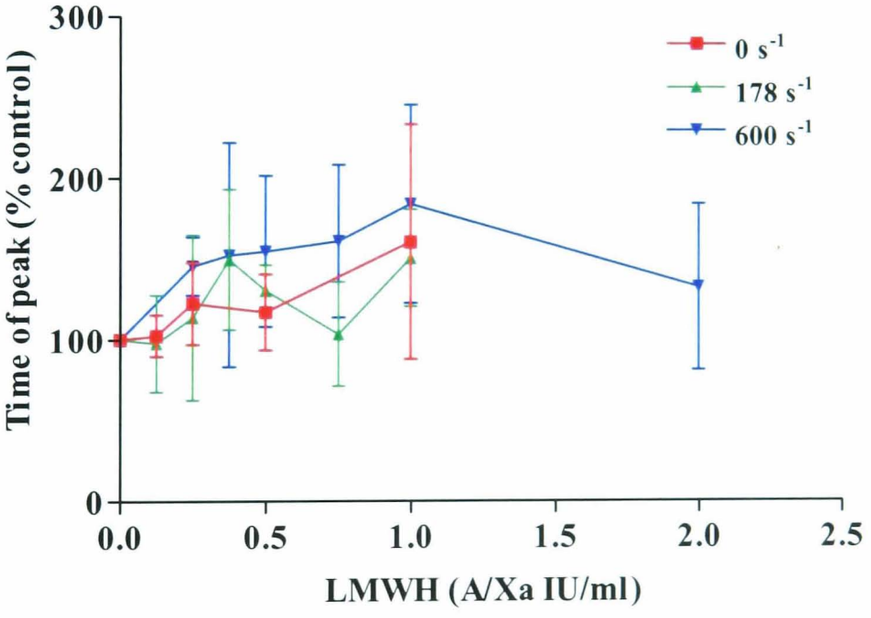

Figure 5.11 *Dose response curves for time of peak thrombin with LMWH.*

800 µl of defibrinated plasma containing 300 x 10^6 platelets was incubated for 30 minutes at 37 °C with 25 µl of TBS containing LMWH. Thrombin generation was initiated in the presence of a) HUVEC or b) ECM. with 175 µl of 100 mM $CaCl_2$ containing recombinant tissue factor to give a final concentration of 35 pM. Timed subsamples were collected into EDTA stop buffer for determination of thrombin concentration with chromogenic substrate S2238.

Fig 5.12a HUVEC

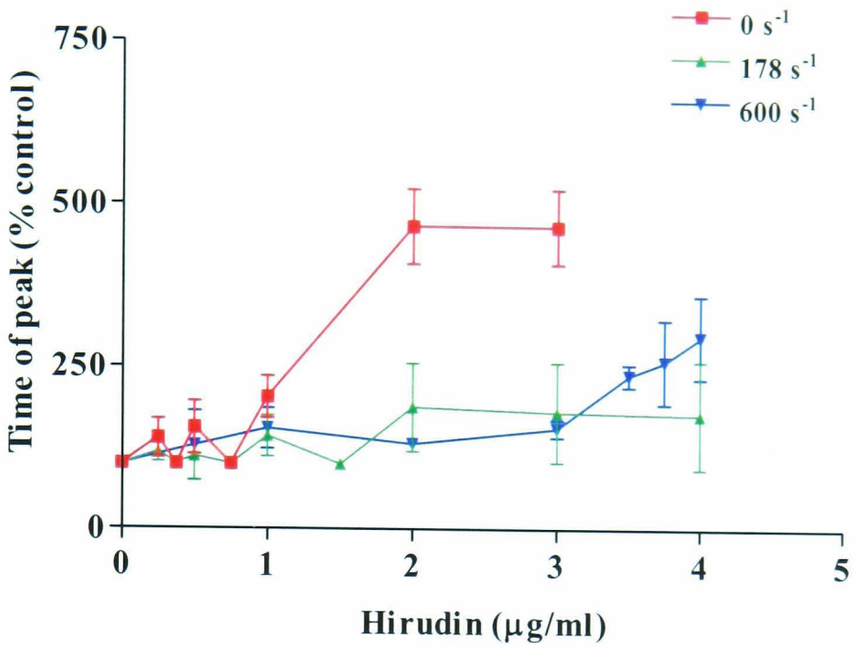

Fig 5.12b ECM

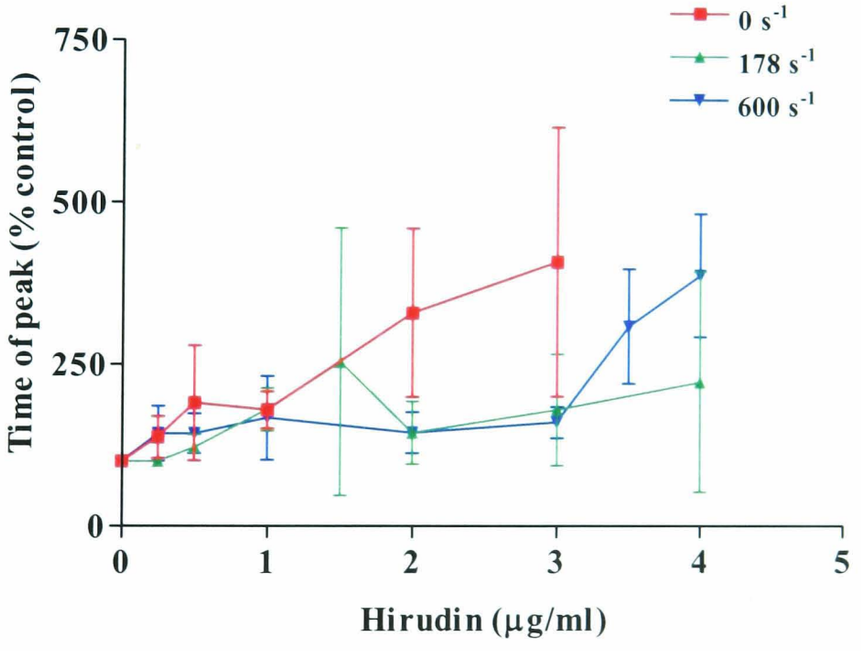

Figure 5.12 *Dose response curves for time of peak thrombin with hirudin.*

800 µl of defibrinated plasma containing 300 x 10^6 platelets was incubated for 30 minutes at 37 °C with 25 µl of TBS containing hirudin. Thrombin generation was initiated in the presence of a) HUVEC or b) ECM. with 175 µl of 100 mM $CaCl_2$ containing recombinant tissue factor to give a final concentration of 35 pM. Timed subsamples were collected into EDTA stop buffer for determination of thrombin concentration with chromogenic substrate S2238.

Fig 5.13a HUVEC + 1μg/ml hirudin

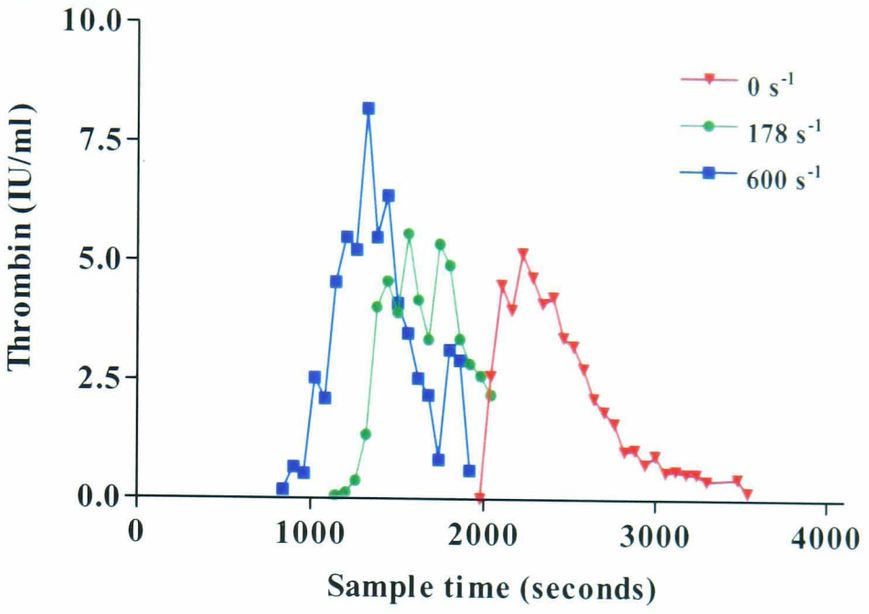

Fig 5.13b ECM + 3μg/ml hirudin

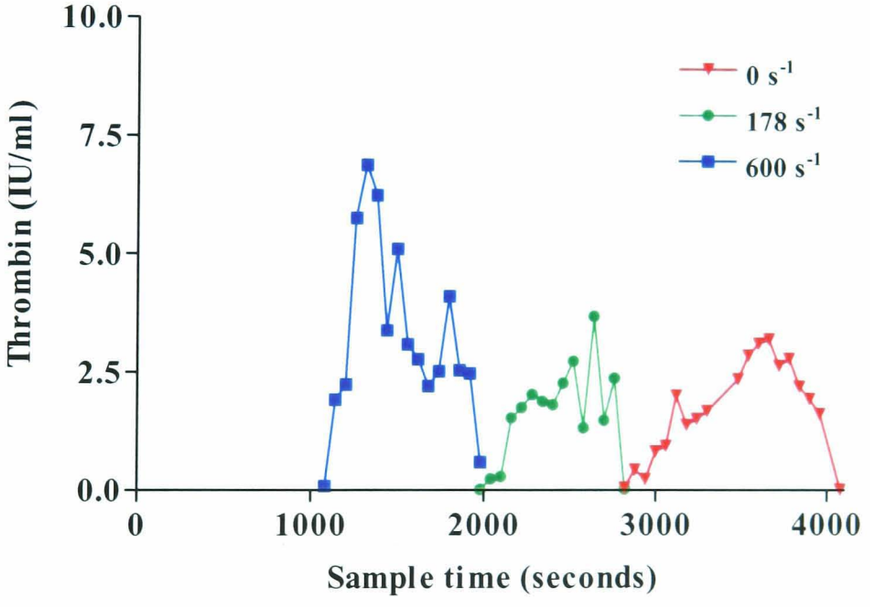

Figure 5.13 *Representative thrombin generation curves from single experiments to show the inhibitory effect of hirudin.*

800 μl of defibrinated plasma containing 300 x 10^6 platelets was incubated for 30 minutes at 37 °C with 25 μl of TBS containing hirudin. Thrombin generation was initiated in the presence of a) HUVEC or b) ECM. with 175 μl of 100 mM $CaCl_2$ containing recombinant tissue factor to give a final concentration of 35 pM. Timed subsamples were collected into EDTA stop buffer for determination of thrombin concentration with chromogenic substrate S2238. The concentration of hirudin was approximately the AUC IC_{50} under static conditions and was 1 and 3 μg/ml in the presence of HUVEC and ECM respectively.

5.3.5 Platelet Factor 4

Assays were performed on subsamples of platelet plasma that had been recalcified only or recalcified and stimulated with TF before being subjected to shear stress at 178 s^{-1}. A control test where the TF/Ca^{2+} stimulated platelet plasma was not subjected to shear stress (0 s^{-1}) was included. No differences were seen between any of the treatments or any of the timepoints assayed (8, 16, 24 and 32 minutes after stimulation) and all of the results were in the region of 700 ng PF4 per ml. A previous experiment had also shown that supernatants from freeze-thawed platelets also contained around 700 ng/ml, and that the background concentration in the absence of platelets and shear stress was around 14 ng/ml.

5.4 Discussion

Unfractionated heparin, low molecular weight heparin and hirudin were studied to define the relative contributions of anti-Xa and thrombin-inhibitory activity to the effectiveness of antithrombotic agents under different physiological conditions.

The APTT assay was used as a simple test to compare the activities of the inhibitors on a weight basis. Hirudin was the most effective inhibitor of the APTT, with twice as much UFH and thirty times more LMWH required to double the blank clotting time. This test was performed in the absence of platelets but in the presence of phospholipid as a platelet substitute and under static conditions, and is a measure of the time taken to generate sufficient thrombin to clot the plasma. The concentration of thrombin required is around 20 nM or 2 IU/ml and is reached early in the propagation phase of coagulation (Hemker, 1994). The propagation phase follows the initiation phase where trace amounts of thrombin have positive feedback effects on the activation of cofactors FV and FVIII leading to the amplified burst of thrombin generation. These results indicate that inhibition of thrombin generated in the initiation phase prevents the positive feedback, delaying the propagation phase and the appearance of the clot. The irreversible and specific binding of hirudin to thrombin appears to be more effective than the catalytic anti-IIa and anti-Xa effects of UFH. The LMWH tested has one-tenth the thrombin-inhibitory activity of UFH when their anti-Xa potencies are equal and, consistent with this, sixteen times more LMWH than UFH was required to double APTT. This indicates that inhibition of thrombin is effective in the inhibition of coagulation under static conditions.

When analysing the data from the thrombin generation studies, the ANOVA statistical analysis allowed the influence of some variables to be accounted for when comparing other variables. Thus it was possible to determine that under static conditions and accounting for the presence of HUVEC or ECM, a significantly higher concentration (on an anti-Xa unit basis) of LMWH than UFH was required to inhibit thrombin generation. The IC_{50} for LMWH was 1.5 times that of UFH, but when these preparations have equal anti-Xa potencies, UFH has 10 times more thrombin-inhibitory activity. This indicates that the thrombin-inhibitory activity of UFH may be responsible for its relatively higher efficacy, but that this is only part of its overall activity and that anti-Xa activity also contributes to the inhibition of thrombin generation. When the data are analysed on a thrombin-inhibitory unit basis, a significantly higher concentration of UFH than LMWH is required, confirming that the higher proportion of anti-Xa activity in LMWH also contributes to its effectiveness. Hirudin was also able to significantly inhibit thrombin generation under static conditions, to a greater degree in the presence of HUVEC than ECM and this was significant in terms of both area under the curve and peak thrombin generation. The specific thrombin inhibitory activity of hirudin is clearly effective under these conditions, and this supports the observations with UFH and LMWH.

The effectiveness of all inhibitors was reduced under flow conditions. As seen in Section 3.3.3.4, compared with static conditions, there was no increase in free thrombin in the presence of ECM under flow conditions. The decrease in the effectiveness of the inhibitors may therefore be due to changes in the interaction of plasma coagulation factors and the inhibitors caused by increased rates of diffusion

in the fluid phase and increased rates of diffusion of substrates and products to and from the boundary layer of the vessel wall under flow conditions (Nemerson, 1995).

The reduced effectiveness of the inhibitors is unlikely to be due to the neutralising action of PF4 released from platelets under shear stress for two reasons. Firstly, PF4 is not an inhibitor of hirudin, and therefore cannot be responsible for hirudin's reduced activity under flow conditions. Secondly, the results of the assays of PF4 release suggest that the release reaction occurred as a result of the TF stimulation, and that shear stress does not significantly influence this. The release of PF4 is not likely to influence the thrombin generation results as the stoichiometry of the PF4-heparin interaction is such that 700 ng/ml of PF4 will neutralise 0.02 IU/ml of UFH, and probably less LMWH due to the reduced charge of the shorter polysaccharide chains (Barrowcliffe *et al*, 1992). This is only 40 % of the very lowest concentration of heparin that was studied in the thrombin generation tests, and despite the possible presence of PF4 this concentration showed a significant inhibitory activity suggesting that PF4 does not have a significant influence in this assay system.

On an anti-Xa unit basis, significantly more LMWH than UFH was required for inhibition under static ($p < 0.01$) and venous ($p < 0.05$) conditions, but under arterial conditions the IC_{50} for UFH and LMWH were similar and the anti-IIa IC_{50} for LMWH was significantly lower than for UFH ($p < 0.001$), indicating the contribution of anti-Xa activity under arterial conditions. This suggests that at higher shear rates, anti-Xa activity becomes more relevant than it was under static conditions and that thrombin-inhibitory activity becomes less important. This hypothesis is supported by

the hirudin data, which show that under flow conditions it was not possible to inhibit total thrombin generation to below 60 % of control levels with thrombin inhibitory activity alone.

Under flow conditions, hirudin was significantly less effective than UFH and LMWH, and UFH was less effective than LMWH in their abilities to reduce peak thrombin generation. This observation supports the theory that anti-Xa activity is more effective in the inhibition of thrombin generation under flow conditions.

The peak concentration of thrombin was seen later in the presence of inhibitors, in a dose-dependent manner, although this was not statistically significant. This increase in the lag time suggests that the feedback activation reactions that lead to the propagation phase of thrombin generation are being inhibited and is consistent with a previous report that found the inhibition of FXa by TFPI reduced thrombin generation in a purified, static system by interfering with the feedback activation of FV (van't Veer & Mann, 1997). The introduction of shear stress did not affect the delay caused by UFH or LMWH, but with hirudin the peak appeared significantly earlier than under static conditions. This suggests that FXa may be playing an important role in the propagation phase of thrombin generation under flow conditions, and therefore a specific thrombin inhibitory agent such as hirudin may be less effective.

Comparison of the activity of heparins and hirudin is very difficult due to their different modes of action (catalysts versus irreversible stoichiometric inhibitor) and the molecular heterogeneity of heparin (making gravimetric and molar comparisons

difficult). Under static conditions hirudin can completely inhibit thrombin generation but under flow conditions it loses this ability, unlike UFH and LMWH. The catalytic effect of the heparins may make them more efficient inhibitors of thrombin generation than hirudin, which may have decreased efficacy because it cannot be recycled – each molecule binds irreversibly to one molecule of thrombin and the hirudin is therefore consumed as thrombin generation proceeds. In addition, the thrombin-inhibitory activity of hirudin may not be as effective an inhibitor under flow conditions compared to anti-Xa activity. Inhibition of FXa, further up the coagulation cascade, reduces the amplification effect of this step and may effectively inhibit the more efficient process of thrombin generation under flow. The delay in the appearance of the thrombin generation peak under static conditions with hirudin suggests that it is inhibiting the trace amounts of thrombin that are generated in the initiation phase of coagulation, thus preventing the positive feedback effects that lead to the propagation phase. The earlier appearance of the peak in flow conditions indicates a decrease in effectiveness – the hirudin may be used up more quickly, possibly due to increased thrombin generation that anti-Xa activity is able to inhibit more effectively.

Previous studies have found that inhibition of thrombin is important in the inhibition of coagulation under static conditions. Two separate studies have shown that TF-stimulated static coagulation *in vivo* was effectively inhibited by UFH but that LMWH, an octasaccharide fraction, or purified pentasaccharide had decreased effectiveness (Buchanan *et al*, 1985; Amar *et al*, 1990). The same was seen in an *in vivo* study where coagulation was stimulated via the intrinsic pathway (Thomas *et al*, 1989). In addition, studies employing venous conditions *in vivo* and *ex vivo* have

demonstrated the effectiveness of anti-Xa agents in the inhibition of thrombosis. *In vivo* TF-stimulated venous thrombosis was inhibited by the specific anti-Xa agent recombinant tick anticoagulant peptide (rTAP) more effectively than by UFH (Vlasuk *et al*, 1991) and *ex vivo* venous thrombosis in response to TF expressed on activated HUVEC was inhibited for a longer period with LMWH than UFH (Diquelou *et al*, 1995b). More recently, a specific direct inhibitor of FXa, DX-9065a was more effective in the inhibition of *ex vivo* thrombus formation under arterial conditions than venous conditions (Shimbo *et al*, 2002), confirming an earlier report of weak *in vivo* inhibition in a venous model (Rogers *et al*, 1999). LMWH has been demonstrated to be more effective than UFH in the inhibition of fibrinogen deposition on injured artery wall (Roque *et al*, 2000), and the antithrombin binding pentasaccharide has proven to be effective in models of arterial thrombosis (Vogel *et al*, 2000). The present results that show an increased role for thrombin-inhibitory activity under static conditions, and for anti-Xa activity under venous conditions, are consistent with these reports.

There are, however, studies that indicate the increased effectiveness of thrombin inhibitory agents over heparin at arterial shear rates. Bossavy *et al* (1998; 1999) have shown that heparin is ineffective at preventing platelet and fibrin deposition onto TF surfaces under arterial conditions, but that hirudin is an effective inhibitor. It must be noted that these experiments employed a shear rate of 2600 s^{-1}, similar to that found in stenosed arteries, whereas our study used a shear rate of 600 s^{-1} which is typical of that found in medium sized arteries. Conversely, a study examining the effectiveness of rTAP under arterial shear conditions showed that selective inhibition of FXa was in fact effective in inhibiting thrombosis on a TF surface at both 600 and

2600 s^{-1} (Orvim *et al*, 1995). It is plausible that the balance between the importance of inhibition of FXa and thrombin may continue to shift as the shear conditions change and that both become significant at extreme shear rates, depending on the parameter that is being measured.

5.5 Summary

The present results show that increasing concentrations of the inhibitors UFH, LMWH and hirudin are required to inhibit thrombin generation as the shear rate is increased. They also show that the effectiveness of the specific thrombin inhibitor hirudin decreased as the shear rate increased, in contrast to the predominantly anti-Xa agent LMWH, which became as effective as UFH at higher shear rates. This suggests that different approaches may be required for the prevention and treatment of different clinical conditions – heparin may be appropriate for venous thrombosis, but arterial syndromes may require the use of predominantly anti-Xa agents. Studies using a direct anti-Xa agent such as DX-9065a and the heparin pentasaccharide would be of interest to examine the contribution of direct over indirect (AT-mediated) FXa inhibition in arterial conditions.

CHAPTER 6

DISCUSSION

6.1 Background

This thesis describes the design and use of a system that enables the study of antithrombotic agents under flow conditions and in the presence of relevant cellular components. The system was based upon that designed in the laboratory of Dr Yale Nemerson (Contino *et al*, 1991) who used capillary tubes to study the influence of flow on the reaction kinetics of coagulation. His theory that the transport rate of substrates and products to and from vessel wall enzymes are the rate limiting steps in coagulation has proved that it is crucial to include flow conditions in these studies, as the transport rates are influenced by the shear rate (Nemerson, 1995). The group published numerous papers, using purified components of the extrinsic tenase complex and glass capillaries to prove the influence of flow on the rate of FXa generation (Gemmell *et al*, 1988; Gemmell *et al*, 1991; Andree *et al*, 1994; Andree & Nemerson, 1995; Gentry *et al*, 1995). This work was continued in the laboratory of Dr Theo Lindhout, who also used a capillary system to study the effects of flow on the assembly and activity of the prothrombinase complex from purified components (Schoen *et al*, 1990; Billy *et al*, 1995b). This group extended their studies to examine the effects of flow on the action of anticoagulants such as heparin and pentasaccharide, showing that antithrombin depletion in the boundary layer limits the effectiveness of these agents at the vessel wall and that their main inhibitory effect is exhibited in the fluid phase (Lindhout *et al*, 1995; Billy *et al*, 1995a). The difficulty in establishing intact monolayers of cells inside capillary tubing has only been overcome once, when Lindhout et al (1992) studied the inhibitory effect of TFPI on the extrinsic tenase complex, finding that even activated endothelial cells were much less procoagulant than the extracellular matrix. A more practical approach to the study of endothelial cells under flow conditions was pioneered by Dr Kjell

Sakariassen, who developed the parallel plate flow chamber that could generate a defined shear rate across a coverslip coated with cells, or other substrates of interest (Sakariassen *et al*, 1983; Sakariassen *et al*, 1984). Among the numerous studies performed in this chamber was the early characterisation of GP Ib/IX/V as an adhesive receptor and GP IIb/IIIa as an aggregatory receptor (Sakariassen *et al*, 1986). The wide range of applications of this and other chambers have been recently reviewed by Sakariassen *et al* (2001).

6.2 The flow system

By combining the principles of Nemerson's flow reactor with the inhibitor studies of Lindhout and the cellular components of Sakariassen, the studies detailed in this thesis aimed to establish a test system for antithrombotic agents that included some of the critical *in vivo* influences that are often missing from *in vitro* assays. The discrepancies that are noted between *in vitro* results and the true *in vivo* situation, such as in the measurement of APTT following heparin therapy (Hirsh *et al*, 2001) may, *inter alia*, be the result of the different reaction rates that are possible in a closed static system and a flow system where fresh reactants are constantly being delivered, and products removed. In addition, the presence of the endothelial cells that line every blood vessel and play an active anti- or pro-coagulant role depending on their state of activation, and of platelets, the blood cell critical to coagulation, were expected to make a major difference to the results obtained.

A number of validation studies were carried out on the flow system to ensure that it performed reliably and to examine the effects of flow on the cellular components of the system. The thrombin generation test was selected as the most suitable method to

measure coagulation in this system, and the effects of flow and the presence of cells were determined. Once the system had been validated and a suitable, physiologically-relevant, platelet-dependent thrombin generation system established, studies were performed to investigate the effects of antithrombotic agents under flow conditions.

6.3 Studies with anti-platelet antibodies

Studies were performed to evaluate the inhibitory effect of antibodies against the two major adhesive receptors of the platelet, GP IIb/IIIa and GP Ibα, part of the GP Ib/IX/V complex. The antibody to GP Ibα was initially included as a negative control, but proved to have significant inhibitory activity which was pursued as an interesting finding. However, further investigation led to the conclusion that this activity was the artefactual result of a contaminant in the original batch of antibody. This suggests that antibodies directed against the vWF-binding function of GP Ibα are not effective in the inhibition of thrombin generation.

The results for the anti-GP IIb/IIIa antibody showed a clear reduction in the antithrombotic efficacy of the antibodies under venous flow conditions compared to static or arterial flow conditions. This is an interesting finding that fits with the accepted theory that arterial thrombosis is mostly mediated by platelets and thus an inhibitor of the major aggregatory receptor on platelets will be effective. There are no similar reports to this in the literature, although an increase in the IC_{50} of small molecule inhibitors of GP IIb/IIIa have been reported in comparisons of platelet aggregation with adhesion to collagen under flow conditions (Mousa *et al*, 2002; Wang *et al*, 2002) and shear-induced platelet adhesion (Abulencia *et al*, 2001). The

clinical experience with antagonists of GP IIb/IIIa also supports the theory – abciximab is effective when used in during percutaneous surgical intervention, but not for any venous conditions. This confirms the utility of the flow system in predicting the *in vivo* efficacy of agents that have proven effective in closed systems under static conditions *in vitro*. In support of this, a flow chamber system has recently been found to be useful in the assessment of the efficacy of antiplatelet agents and an anti-GP IIb/IIIa antibody on stent-induced thrombosis (Sakakibara *et al*, 2002).

6.4 Studies with heparin and hirudin

Platelets play a key role in primary haemostasis, adhering and aggregating to seal breaches in the vessel wall. These processes are mediated by platelet membrane glycoprotein receptors, and antagonists of these receptors may interfere with both their adhesive function and the outside-in signalling that would activate the platelet, as has been noted with anti-GP IIb/IIIa agents (Reverter *et al*, 1996; Pedicord *et al*, 1998; Herault *et al*, 1998). The second phase of haemostasis is the development of a stable fibrin clot, following activation of the coagulation cascade, a process to which platelets contribute by the release of coagulation factors and the expression of a catalytic surface for their interaction. The most potent activator of platelets is thrombin, generated in the initiation phase of coagulation, which has an explosive positive feedback effect on coagulation. An alternative approach to the inhibition of thrombosis is therefore to inhibit the generation of thrombin through the use of anticoagulants, and studies were performed to assess the effects of different flow conditions on heparin and hirudin.

The anticoagulants heparin and hirudin were also seen to have reduced effectiveness in the inhibition of thrombin generation under flow conditions. Clear differences were seen between unfractionated and low molecular weight heparin, and these changed with the shear rate. Under static and venous conditions, significantly more LMWH than UFH was required for inhibition, as was seen in the APTT assay, but this difference was eliminated under arterial conditions. This suggests that the anti-Xa activity of the compounds increased in importance as the shear rate increased. Although the direct thrombin inhibitor hirudin is not strictly comparable with heparin, its effectiveness in the inhibition of thrombin generation under static conditions and in the APTT, coupled with its inability to inhibit thrombin generation to any great extent under flow conditions, supports the hypothesis that anti-thrombin activity is less relevant under conditions of increased shear stress. These findings are consistent with previous reports that have found UFH to be more effective than LMWHs in models of *in vivo* stasis (Buchanan *et al*, 1985; Thomas *et al*, 1989; Amar *et al*, 1990), and studies that showed anti-Xa agents to be effective in the inhibition of *in vivo* and *ex vivo* venous thrombosis (Vlasuk *et al*, 1991; Diquelou *et al*, 1995b). This again illustrates how the flow system can provide data that is relevant to the *in vivo* situation without the need for invasive procedures. However, a limitation of the flow system as a model of *in vivo* coagulation may be revealed when analysing the hirudin results. One of the benefits of hirudin is its ability to inhibit clot-bound thrombin and thus prevent expansion of an existing thrombus. This is a clinical situation that may not be adequately represented in these experiments, although future work could include experiments with fibrin clots generated on coverslips. Hirudin is also a more effective inhibitor of fibrin deposition and platelet adhesion than heparin (Bossavy *et al*, 1999) due to its

irreversible inhibition of thrombin, a major activator of platelets. Clinically, UFH and LMWH have traditionally been used for the treatment and prevention of venous thrombosis, but are increasingly used for treatment of acute coronary syndromes (Hirsh *et al*, 2001). Further confirming the potential of anti-Xa agents in arterial conditions, the new pentasaccharide drug Fondaparinux is entering phase III clinical trials for use in acute coronary syndromes, as well as for venous thromboembolism (Lensing, 2002).

6.5 Conclusions

The results presented in Chapters 4 and 5 allow a theory to be formulated regarding the process of coagulation under flow conditions, in contrast with the static studies, and outlined in Figure 6.1. Coagulation was stimulated with a low concentration of TF to mimic low-level damage within the vasculature. The inhibition of GP Ib/IX/V at one of its vWF adhesion sites was not effective in the inhibition of coagulation under these conditions, but inhibition of GP IIb/IIIa was particularly effective under static or arterial conditions, illustrating the importance of flow in the consideration of *in vitro* data. The efficacy of the anti-GP IIb/IIIa antibody at arterial shear rates suggests that platelet-platelet interaction is important in the amplification of the response, but also that platelet activation via GP IIb/IIIa may be important in the development of the platelet-mediated coagulation response. The efficacy of anticoagulants was also markedly reduced under flow conditions. Under arterial conditions coagulation was most effectively inhibited by anti-Xa agents and direct or indirect inhibition of thrombin was less effective. The results suggest that under arterial conditions, the assembly and activity of the prothrombinase complex is the

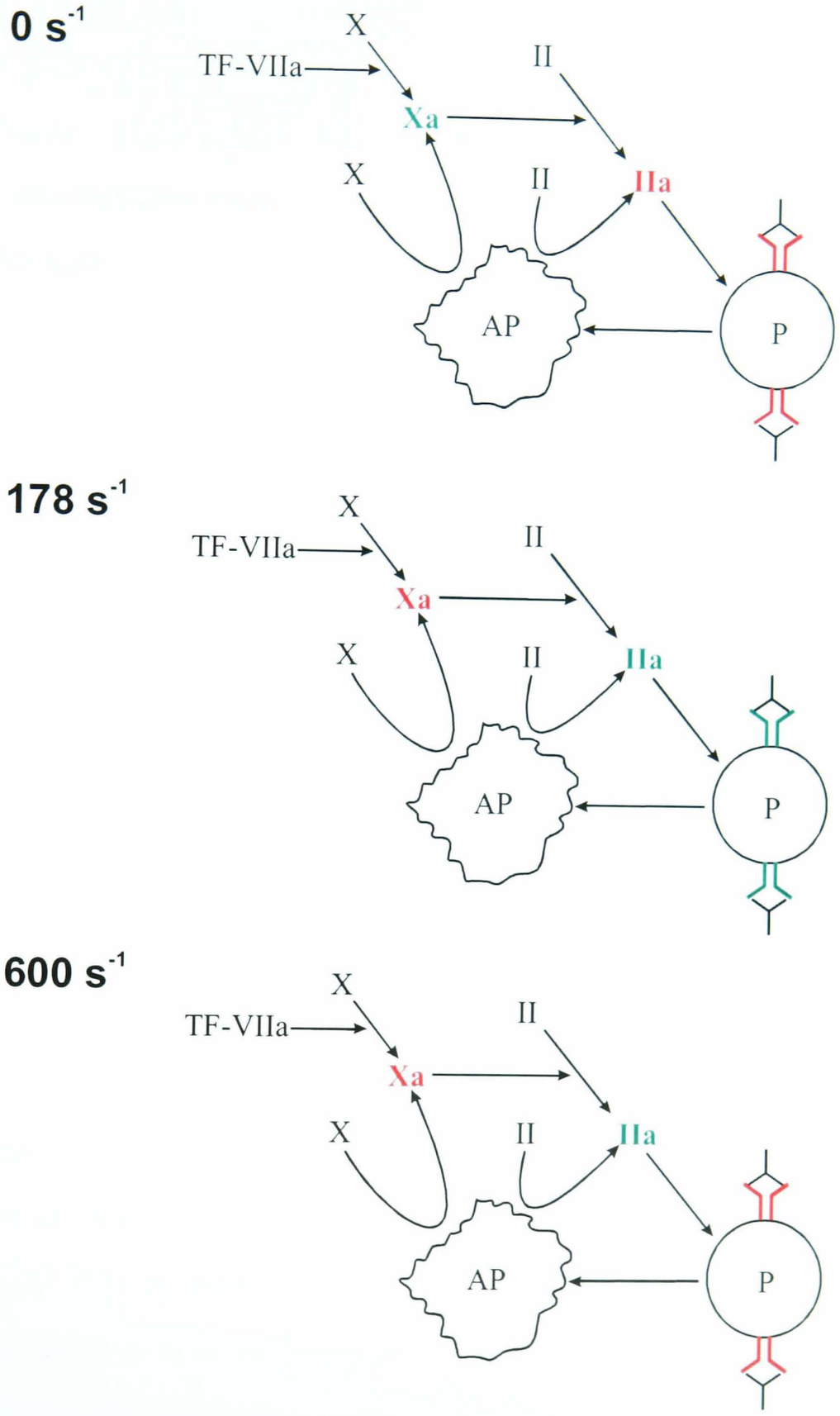

Figure 6.1 *A simplified theory of the inhibition of coagulation under flow conditions*

Red *indicates targets of strong inhibition; green indicates targets of weak inhibition. P = resting platelet (with GP IIb/IIIa and a bound inhibitory antibody shown), AP = activated platelet.*

critical stage of coagulation and that inhibition of free thrombin is of limited effectiveness. Under venous conditions, inhibition of platelets via GP IIb/IIIa is weak, and coagulation is more effectively inhibited by anti-Xa agents than by anti-thrombin agents.

6.6 Future work

The flow system used in this study does have some limitations, which may be addressed in future work. The design of the flow chamber made it difficult to study the adhesion of platelets to the endothelial cells or their matrix. This would have been useful, as the antibodies used are against adhesive receptors (although the antibodies are still able to prevent activation via the receptors), and platelet adhesion plays an important role in the coagulation process. These studies were primarily concerned with the 'end-product' of the coagulation process, measured as the generation of thrombin, but it would have been helpful to visualise the interactions that were occurring at the 'vessel wall'. Post-perfusion analysis of the coverslips was also more difficult than had been hoped, as the coverslips were liable to stick to the gasket and then slip across it when the flow chamber was dismantled, thus removing any cells before microscopy. Redesign of the chambers may allow *in situ* microscopy, if the top block was thinner and a transparent base was used, and this should not prove to be an overly expensive commission. Real-time microscopy of the perfusion, with image capture equipment would allow studies of the interaction of platelets with the (sub)endothelium, and allow the process of clot formation, and how inhibitors affect it, to be studied dynamically. In addition, the use of fluorescent labels on antibodies, inhibitory agents or tissue factor would allow their detection

during the course of a perfusion, and the assembly of the clot could be visualised *in situ* – arrangements are in progress for a rheological microscope stage to be modified for this purpose. Alternatively, the DiaMed Impact (DiaMed, Cressier sur Morat, Switzerland) is a new diagnostic device that analyses the adhesive capacity of platelets in whole blood, using a cone and plate system to apply shear stress and providing image analysis and quantitation. This machine has potential for rapid assessment of the anti-adhesive capacity of inhibitors using different substrates and shear rates, and could be of use alongside the flow system for the analysis of coagulation under matched conditions. Contacts have been made with the manufacturer and a collaboration is being arranged.

The absence of red blood cells, leukocytes and fibrinogen and the presence of citrate detract somewhat from the physiological relevance of the system, but all are necessary for practicality. The ideal system would allow studies with non-anticoagulated whole blood. This would incorporate the physical interactions between red cells and platelets, the contribution of red cells to coagulation, the potentially pro-inflammatory and pro-coagulant interaction of platelets with leukocytes, and the roles of fibrinogen in platelet aggregation and coagulation. However, if whole blood were to be used the red cells would interfere with the detection of thrombin by a chromogenic substrate. In addition, the red cells would also interfere with the real-time microscopy studies suggested above. The use of a fluorogenic substrate for thrombin detection is a potential solution to the interference of red cells with chomogenic substrates, but strict standardisation would be required as the haematocrit would still have a strong influence on the results. It would be

difficult to perform a thrombin generation test in parallel with this system, but it may be possible with manual sampling in an anticoagulated system.

Another area that could be standardised in future studies is the surface area to volume ratio of the plasma and the cells. This was directly comparable in the venous and arterial experiments, but different for the static and low shear (18 s^{-1}) experiments, as a result of the design of the Type 2 flow chambers that were built to maximise the length of the experiments where volume of perfusate was the limiting factor, and recirculation was not desirable. Due to the anticoagulant nature of the endothelial cells and the procoagulant nature of the extracellular matrix, the volume of plasma that is in contact with them and the duration of contact will have an effect on the coagulation that is proceeding. This difference may be partly responsible for the reduced effectiveness of the inhibitors under static conditions in the presence of ECM compared to HUVEC, and the lack of difference when shear stress was applied, suggesting either that the reduced surface area available for contact with the plasma was having a lesser effect on coagulation, or that the cells were becoming procoagulant. Further studies to determine the activating effect of short-term shear stress on HUVEC will be needed to answer this question fully.

Other agents that would be interesting to study in the flow system include antibodies against the other adhesive and activating receptors on the platelet membrane such as GP Ia/IIa, GP VI and other epitopes on GP Ibα such as the thrombin binding site. It would be particularly interesting if a combination of antibodies against a number of receptors used together could completely inhibit platelet activation in an additive manner, as has been seen to some extent with antibodies to GP IIb/IIIa and GP Ibα

(Wu *et al*, 2002a). Studies of direct anti-Xa agents such as DX-9065a and rTAP would allow direct comparison with hirudin, and studies with the pentasaccharide would add to the UFH and LMWH data. Combination studies would also be interesting, as many patients do not receive a single drug in isolation. The majority of at-risk patients take aspirin or clopidogrel routinely, and the flow system is particularly suited to the study of combined anticoagulant and antiplatelet agents as it incorporates both platelet and plasma components of coagulation.

6.7 Summary

The results presented indicate clear differences in the mode of action of antithrombotic agents under flow conditions. The experimental conditions used were an approximation of clinical situations where coagulation is initiated at one point in the vasculature and the activated blood is washed downstream, passing over intact or damaged endothelium where the extracellular matrix is exposed. The effect of this (non-) reactive surface on the progress of coagulation is clearly important, for instance in invasive procedures where endothelial damage is inevitable and antithrombotic agents are used to prevent occlusive thrombi forming. The results that were obtained show significant differences in the efficacy of antithrombotic agents under a variety of physiological conditions and that this flow system is capable of providing data relevant to the *in vivo* situation.

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APPENDIX 1: α_2M-THROMBIN CALCULATION

The method used to calculate the amidolytic activity of the α_2M-thrombin complex followed the methods of Hemker *et al* (1986). The following explanation and the computer programme used to automate the calculation procedure were both written by Peter Rigsby, Informatics Laboratory, NIBSC, UK.

Let $A(t)$ denote the total amidolytic activity at time *t*, $T(t)$ denote the thrombin concentration at time t and $\alpha(t)$ denote the $\alpha_2 m$-thrombin concentration at time *t*. The relationships $\frac{d}{dt}\alpha(t) = k_2 T(t)$ and $A(t) = T(t) + f\alpha(t)$ are then solved, for the discrete case $(t_1, t_2, t_3 ...)$, with constants k_2 and f as described by Hemker *et al* (1986). The area under the *T(t)* curve is then calculated – again for the discrete case.

APPENDIX 2: FLOW SYSTEM SOFTWARE

The syringe pump and XYZ-translation table of the flow system was controlled by a personal computer running software written by Dr Paul Contino of the Department of Medicine, Mount Sinai School of Medicine, City University of New York, USA. (Contino *et al*, 1991). This software was kindly donated by Dr Yale Nemerson and was adapted for use with the particular models of pump and table by the suppliers of the table, Unimatic Engineers, London UK.

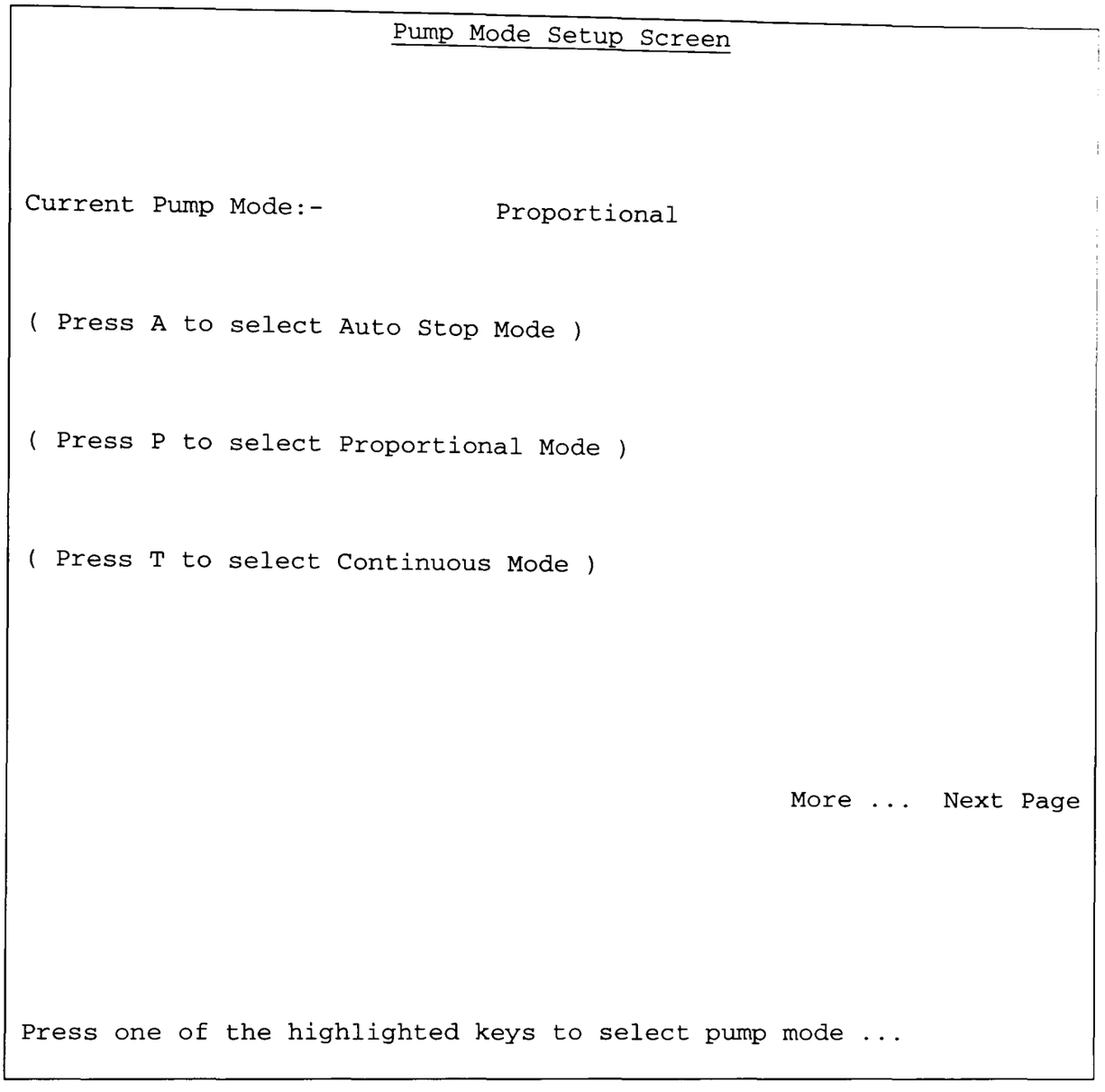
Print outs of the user interface are found on the following pages.

```
Main menu ...

    Origin is set at (X, Y position in mm):      80.0, 77.0
    Upper/Lower limits are (mm):     Upper limit =      62.0
                                     Lower limit =      72.0



    1  START (PLATE TEMPLATE).
    2  START (COBAS TEMPLATE).          -     Not active in this version
    3  Modify origin.
    4  Modify Upper/Lower limits.
    5  Set Pump & Experiment Parameters.
    6  Save present settings.
    7  Get settings from disk.
    8  Set up table parameters          -     Not yet active


Press one of the highlighted keys to perform action ...
```

```
Set origin ...

    Co-ordinate in X direction:      80.0 mm
    Co-ordinate in Y direction:      77.0 mm

    Move to indicated X, Y position

    Finetuning:
       1 X direction
       2 Y direction

    Origin set correctly (Center of well A1).  Continue.

Press one of the highlighted keys to perform action...
```

```
Set Upper/Lower limits ...

   CAUTION: Upper limit is the level at which the arm moves from one
   well to another, and Lower limit is the level to which the arm
   lowers when collecting samples.

   As the <reset> position of the arm in the Z-direction is the
   HIGHEST position of the arm, and the arm moves downwards, the
   distance to the Upper level is smaller than the distance to the
   Lower level.  Therefore under all conditions Upper limit should
   be smaller than the Lower limit !

    The software is safeguarded for this.



Press any key to continue ...
```

```
Set Upper/Lower limits ...

    Upper limit:   62.0 mm

    Lower limit:   72.0 mm

    Move to, and finetune on:  1 Upper limit

                               2 Lower limit

    Upper/Lower limit set correctly.  Continue.

Press one of the highlighted keys to perform action ...
```


Pump Mode Setup Screen
Current Pump Mode:-
Proportional
(Press A to select Auto Stop Mode)
(Press P to select Proportional Mode)
(Press T to select Continuous Mode)
More ... Next Page
Press one of the highlighted keys to select pump mode ...

Setup Pump 2

Number of wells is calculated at :- 32

Use a start value of "0" if syringe 2 not used

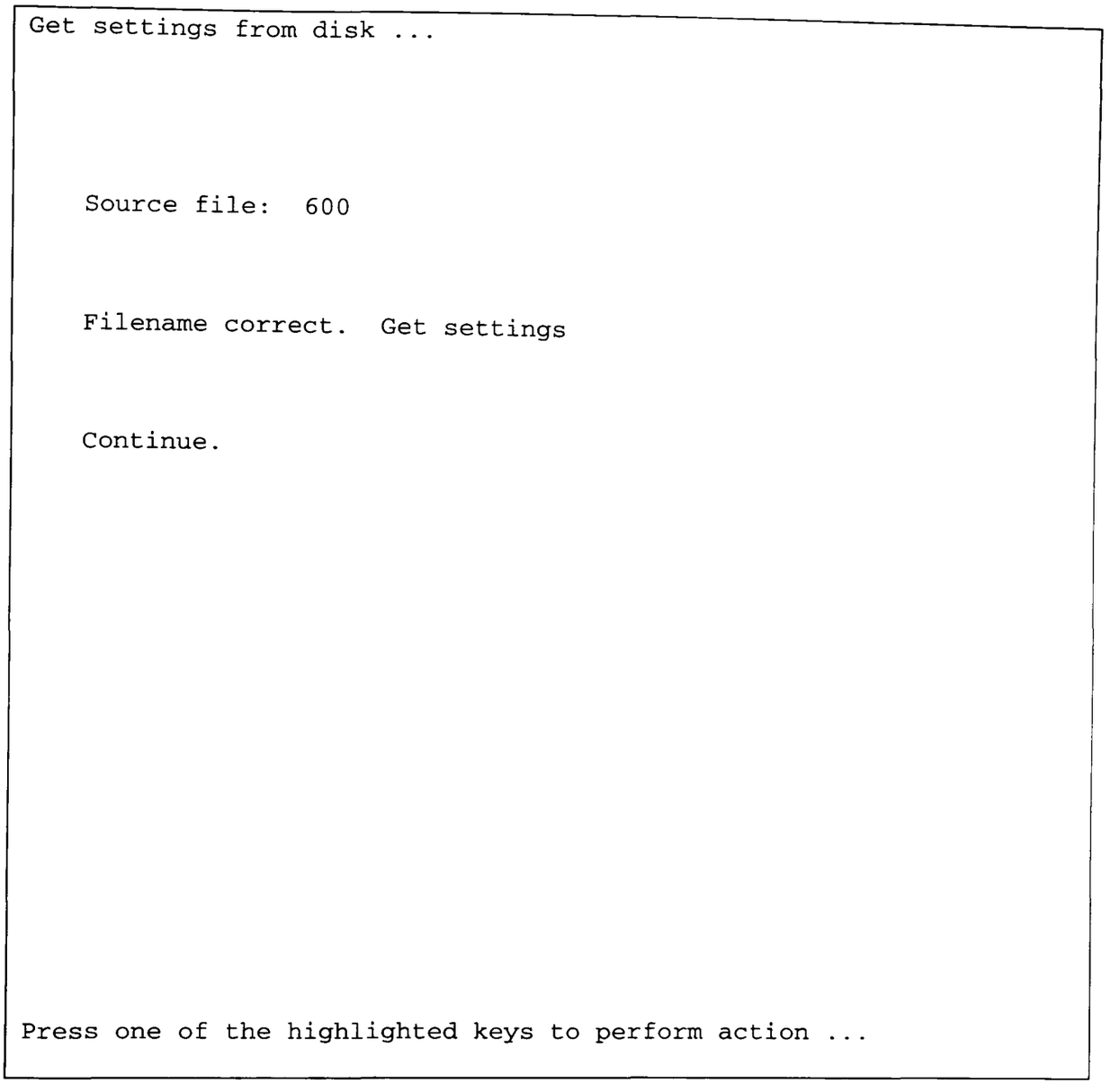
Start syringe 2 at well number 1 - Enter a number between 0 and 96

Stop syringe 2 at well number 32 - Enter a number between 0 and 96

Continue?

Press one of the highlighted keys to select pump mode ...

```
Save present settings ...


    Save as: 600


    Filename correct.  Perform save action.


    Continue.









Press one of the highlighted keys to perform action ...
```


Get settings from disk ...
Source file: 600
Filename correct. Get settings
Continue.
Press one of the highlighted keys to perform action ...

```
X_Y_Z TABLE …

Press <space bar> to stop    NB ! - Only acts at end of a well

Current well no.   1          Estimated experiment time:   32 min.
Time remaining (Secs)         Approx Time Remaining,  Mins,  Secs
          60                                            32

Flow rate "1":   30.0 µl/min

Flow rate "2":   30.0 µl/min - ONLY between Well No 1 & No 32
inclusive

Total number of wells will be:  32

You will be collecting 60.0  µl/well
```